hnRNP A2 is a Protein Involved in the Trafficking Pathway of HIV-1 Genomic RNA

by

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ABSTRACT

HIV-1 genomic RNA trafficking is an important event which remain unclear. Different cellular proteins are involved in this process and the heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) is suggested to play a role by its potential implication in ribonucleoprotein complex which is related with the microtubule network. We identified heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) is involved in this process. To investigate the potential function of hnRNP A2 in the trafficking, protein expression was decreased using small interfering RNA and the impact on RNA localization, protein expression patterns and levels of genomic RNA in new virions was looked at. The results obtained suggested that hnRNP A2 knockdown impedes transport of the genomic RNA and causing its accumulation at the MTOC (microtubule organizing center). These data show the importance of the hnRNP A2 protein in genomic RNA trafficking and its role in HIV-1 RNA localization.

RÉSUMÉ

Le transport de l'ARN genomique est l'une des étapes importantes du cycle virale. Bien que plusieurs protéines cellulaires soient connues pour y jouer un rôle, la protéine hnRNP A2 (*heterogeneous nuclear ribonucleoprotein A2*) serait impliquée dans ce processus par son implication dans la formation de complexe ribonucléoprotéique interagissant avec le réseau de microtubule. Nous suggérons alors que hnRNP A2 serait impliquée dans ce processus. Pour évaluer le rôle de hnRNP A2, nous avons réduit son expression protéique, puis nous avons etudié l'impact potentiel du traitement sur la localisation de l'ARN génomique, l'expression des protéines et finalement, nous nous sommes penchés sur le niveau de l'ARN encapsidé par les nouveaux virions. Les résultats suggèrent que la diminution d'expression de hnRNP A2 influence négativement le transport le l'ARN génomique et provoque une accumulation au centre d'organisation des microtubules. Ces analyses suggèrent donc un rôle de la protéine hnRNP A2 dans le transport et la localisation de l'ARN.

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PUBLICATIONS

1. Bériault V, Clément JF, Lévesque K, Lebel C, Yong X, Chabot B, Cohen EA, Cochrane AW, Rigby WF, Mouland AJ. A late role for the association of hnRNP A2 with the HIV-1 hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. J Biol Chem. 2004; **279** (42):44141-53

2. Levesque K, et al. HnRNP A2 mediates the trafficking of HIV-1 genomic RNA form the microtubule organizing center toward the plasma membrane. Manuscript in preparation.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

3TC	2',3'-dideoxy-3'-thiacytidine; lamivudine
A2RE	hnRNP A2 response element
a.a.	amino acid
ALV	avian leucosis virus
ASH1	achaete-scute homologue 1
AZT	3'-azido-2'-deoxythymidine; zidovudine
AIDS	acquired immunodeficiency syndrome
BIV	bovine immunodeficiency virus
BLV	bovine leukemia virus
Bp	base pair
CA	capsid of HIV-1; p24
CaMKIIα	α subunit of Ca2+ / calmodulin-dependent protein kinase II
cDNA	complementary DNA
CPEB	cytoplasmic polyadenylation element binding protein
CD	cluster of differentiation
CDK9	cycline-dependant kinase family 9
CRM 1	chromosome region maintenance 1
CTE	constitutive transport RNA element
CTL	cytotoxic T lymphocyte
Cyp A	cyclophilin A
DC-SIGN	dentritic cell-specific intercellular adhesion molecule grabbing nonintegrin
DIS	dimer initiation site
dsRBP	double-stranded RNA-binding protein
ECL	enhanced luminol-based chemiluminescence
Env	envelope
eIF5A	Eukaryotic translation initiation factor 5A
EIAV	equine infectious anemia virus
ER	endoplasmic reticulum

ESCRT-1	endosomal sorting complexes required for transport
FeLV	feline leukemia virus
FIV	feline immunodeficiency virus
Gag	group specific antigen
GFP	green fluorescent protein
GLV	Gibbon ape leukemia virus
GRD	glycine-rich domain
HIV	human immunodeficiency virus
hnRNP	heterogeneous nuclear ribonucleoprotein
hPOSH	human plenty of SH3s
hRip	human Rev-interacting protein
H.A.A.R.T	highly active anti-retroviral therapy
HSP	heat shock protein
HTLV	human T-cell leukemia virus
ICAM-1	intercellular adhesion molecules type 1
IN	integrase of HIV-1; p31
IFN	interferon
kb	kilobase
Kd	dissociation constant
kDa	kiloDalton
LAV	lymphadenopathy-associated virus
LFA-1	leukocyte function associated molecule -1
LTNP	long term non-progressor
LTR	long terminal repeat
MA	matrix of HIV-1; p17
MAPK	mitogen-activated protein kinases
MBP	myelin basic protein
MBV	multivesicular bodies
MHC1	major histocompatibility complex
MHR	major homology complex
MLV	murine leukemia virus

µg	microgram
µl	microliter
µM	micromolar
µg	microgram
mM	millimolar
mL	milliliter
Mo-MuLV	Moloney murine leukemia virus
MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA (ribonucleic acid)
MTOC	microtubule organizing center
ng	nanogram
nm	nanometer
NC	nucleocapsid of HIV-1; p7
Nef	negative regulatory factor of HIV-1; p27
NF-kB	Nuclear factor-kappa-B
NNRTI	non-nucleoside analogue reverse transcriptase inhibitors
NRS	nuclear retention signal
NRTI	nucleoside analogue reverse transcriptase inhibitors
nt	nucleotide
NTF2	nuclear transporter signal 2
ORF	open reading frame
PKR	dsRNA-dependent serine/threonine protein kinase
Pol	polymerase
PPT	polypurine tract
PBS	phosphate buffered saline
PBS	primer-binding site
PIC	pre-integration complex
PR	protease of HIV-1; p11
psi	packaging signal; ψ
Rab	Ras-associated small GTPases
Rev	regulator for gene expression of HIV-1; p19

RILP	Rab7-interacting lysosomal protein
RNA	ribonucleic acid
RNP	ribonucleoprotein
RRE	Rev-responsive element
RRM	RNA recognition motif
RSV	Rous sarcoma virus
RT	reverse transcriptase of HIV-1; p51/p66
RTC	reverse transcription complex
SU	surface envelope glycoprotein of HIV-1; p14
Sqd	Squid protein, hnRNP homologue in *Drosophila*
TAR	transactivation response element
Tat	transcriptional transactivator of HIV-1; p14
TCE	translational control element
TFIID	transcription factor IID
TM	transmembrane envelope glycoprotein of HIV-1; gp41
TNF	tumor necrosis factor
TRBP	TAR RNA-binding protein
TRIM5α	tripartite interaction motif 5α
Tsg101	tumor susceptibility gene 101
UTR	untranslated region
VAN	virion-associated matrix-interacting protein
Vif	viral infectivity factor of HIV-1; p23
VLP	virus like particle
Vpr	viral protein R of HIV-1; p15
Vps	vacuolar protein sorting
Vpu	viral protein U of HIV-1; p16

Chapter 1 INTRODUCTION

This thesis will start with an overview of general concepts concerning HIV-1. Different aspects of this virus will be described such as general structure and organization, replication cycle and interaction with cellular proteins. The concept of RNA trafficking will be explored and described in the context of viral infection. Work done in our laboratory and in the field will be described. Finally, the objectives fixed for this research project will be described.

1.1 What is HIV ?

Human immunodeficiency virus (HIV), is the causal agent of the acquired immunodeficiency syndrome (AIDS) and belongs to the family of retroviruses. HIV is especially lethal because it attacks the immune system cells which are responsible for defending the organism against infections.

1.1.1 Discovery and History

In 1981, a new, lethal syndrome characterized by a state of severe immunodeficiency leading to severe opportunistic infections such as *Pneumocytis carinii* pneumonia as well as uncommon neoplasms such as the Kaposi sarcoma[1, 2], was first described in young homosexual males. This new clinical entity was named Acquired Immunodeficiency Syndrome (AIDS). The immunological impairment at the basis of AIDS was a deficit in the CD4+ T lymphocytes compartment[3, 4]. Epidemiological studies showed that the disease was transmitted between individuals through bodily fluid exchange, in particular through sexual acts or blood contact. Further analysis led to the hypothesis that the disease was caused by a virus of the retroviridae family, which was thought to be similar

to the human T-cell leukemia virus (HTLV). It was initially named HTLV-III, due to the similarity to HTLV, and also with the lymphadenopathy-associated virus (LAV). In 1983-1984, several investigators, such as the team of Luc de Montagnier from (France) and Robert Gallo from (United States of America), worked to identify and characterize the virus. This effort led to the isolation and description of the Human Immunodeficiency Virus (HIV) as the aetiologic agent of AIDS [5, 6].

1.1.2 Transmission and symptoms

HIV-1 epidemic is steadily increasing in the developed countries, while it has reached the proportion of a pandemic in developing countries. In 2004, around 40 million individuals is estimated to be infected worldwide, but some estimates based on the rate of progression of the disease show that by 2010 the number of infected individuals in Russia, China and India alone will be around 40 million[7, 8].

The virus can be found in biological fluids of an infected individual. However, not all biological fluids have the same risk of transmission; the viral concentration is the main determinant of this risk. Because the virus concentration tends to be higher in the blood, contact with a small quantity might be enough for infection. In the case of other biological fluids, such as the pre-seminal fluid, a higher amount is usually required for HIV-1 transmission. There is no evidence to date of HIV-1 transmission through saliva, sweat or urine. However high its charge, the virus can't infect an individual until it overcomes the body's natural physical barriers, such as the skin[9]. Two major routes of transmission are thoroughly described and accepted: sexual and non-sexual. The first one is explained as the contact with vaginal, seminal or blood fluids. The mouth could also be considered a route of infection during certain sexual acts. The second one is related to blood transfusions, vertical transmission from mother-to-child, and use of contaminated needles from intravenous drug users.

After becoming infected, the person's immune system will fight the agression and will show symptoms similar to an influenza infection and may suffer from glandular fever [10]. After a few weeks, the newly infected individual will develop antibodies which can be detected in an average of twelve weeks post-infection[11].

1.1.3 Cellular responses to HIV-1

Pathogens are found everywhere and as soon of they have an opportunity, they will establish themselves in a proper environment which allows them to survive and replicate. Unfortunately, the human body is an ideal environment in which to thrive. In fact, our body is attacked by pathogens on a daily basis. Immunological activation is then required and essential in the response to invading organisms. Like other pathogens, HIV-1 will induce cellular activation by different mechanisms, leading to the production of antibodies that will be directed against the pathogen. But, HIV-1 will use and take advantage of the host machinery, to survive and allow viral replication [12].

The onset of HIV-1 infection is hard to detect since the symptoms are similar to influenza. Once the immune system responds to the infection, HIV-1 antibodies will be detectable. Described to have a cellular tropism for CD4 receptor, HIV-1 is well known to infect cells such as macrophages, monocytes, T helper lymphocytes [13, 14]. Once infected, the organism will initiate cellular responses, leading to the activation and secretion in the blood of different factors, such as soluble TNF II receptor, and different cytokines like IFN-γ, TNF-α and IL-1β. The acute presence of these factors will induce activation of cytotoxic T lymphocyte (CTL) CD8+cells, playing an essential role in primary infection[15]. A few weeks following infection, the humoral response will take place and allow production of antibodies in response to this specific pathogen which will help clear the infection [16]. However, the continuous hyper-replication of HIV-1 and the constant immune system response will promote the presence, within the viral genome, of neutral mutations, drug resistant mutations and genetic drift, all of which will contribute to the overall antigenic variability of the virus. Because of this constant evolution of the

HIV-1 virus, antibodies produced to target a specific epitope may not be effective against a new variant of the virus [17].

Once the initial viral infection is cleared up after the onset of the humoral immune response, HIV-1 will persist in viral reservoirs, such as macrophages and dendritic cells, allowing the continuation of the viral cycle [18-20] This will lead to development of a chronic infection associated with a clinical latency which does not imply viral latency. During this time, presence of the viral envelope glycoprotein gp120 will activate secretion of TNF-α and will support viral replication [21] by acting on NF-kappaB, a transactivator of the viral LTR [22]. Promotion and continuation of viral replication will lead to a gradual decline of the number of CD4+ lymphocytes cells, involved in cytokines production that stimulate B lymphocytes and antibodies secretion, until it reaches a critical point where cell-mediated immunity is lost. At this point there is no longer an effective immune response towards infections usually kept under control by our immune system, such as *mycobacterium tuberculosis*, *Candida* and influenza. These infections will develop unabated, eventually leading to death [23].

Most people that will be infected by HIV-1 will have the normal disease development pattern described above. However, some individual who have been living with HIV-1 for at least 7 to 12 years, present relatively no symptoms and a low viremia. These individuals are known as long term non-progressors (LTNP). LTNPs are currently subject of different studies to understand the cause of the low disease progression associated with a low viral load even if no treatment was given. Different hypotheses have been elaborated. One of them is based on the hypothesis that this phenomenon is associated with the maintenance of the integrity of the lymphoid tissues and with less virus trapping in the lymph nodes than is seen in other individuals with HIV-1. However, it is also thought that it may be related to the presence of different mutations in essential viral genes, such as the viral protein R (Vpr) or the envelope (Env gp160) [24, 25].

1.1.4 Treatment

Immunosuppressive drugs, such as thalidomin, pentoxifyllin or cyclosporin A, were first given to patient [26] to target and reduce activation of lymphocytes and TNF-α, except that no efficient results were obtained. Then attempts were made to treat the infection with different drugs that would target specific enzymes of HIV-1 such as protease (PR) or reverse transcriptase (RT). Initially, a single target drug trial was used, usually targeting the reverse transcriptase enzyme [27]. However, the continual creation of new mutations within the HIV-1 genome, which affect either positively or negatively the fitness of the virus, was a major issue in regards to drug resistance. Rapid replication of the virus made it difficult to predict, to treat and to eradicate. The continuous viral replication at the level of 10^9-10^{10} viruses per day, the low fidelity of RNA polymerase and the deficiency in proofreading mechanisms, used in detecting and correcting potential mutations, are all important factors leading to creation of mutations at an average of $3,4 \times 10^{-5}$ nucleotides [28].

Different drugs may be combined together and used in a more efficient way to avoid the rapid failure of the monotherapy. Today's treatment are a combination of two nucleoside analogues (NRTI) and one protease inhibitor (PI), which will reduce the viral load of the patient. Also known as the highly active anti-retroviral therapy (H.A.A.R.T), it acts on the retroviral replication by interfering with different essential enzymes, such as the RT and PR which will eventually decrease the viral load and have positive effect on the CD4+ cell levels, which may be stabilized [23]. Like other therapies, it also involves risk of drug resistance. However, certain mutations can have a positive effect. In fact, one of the most famous mutations present in 3TC treatment, M184V, will have an opposite effect combined with the AZT treatment, by compromising the removal of chain-terminating nucleotides and diminishing the resistance to this drug [23, 29].

Ever since HIV-1 developed resistance to drugs, understanding the role of virus-host interaction becomes more important for the future development of anti-viral therapies.

Therapies which target virus-host interactions is a relevant subject and it has been proposed that emergence of potential mutations could be lower, because the presence of mutations in the cellular machinery may interfere with either the cell and the viral cycle no one will get any benefit. To clarify the existing relation between virus and host, several studies are currently being conducted to identify the potential role of cellular proteins in the viral process [30].

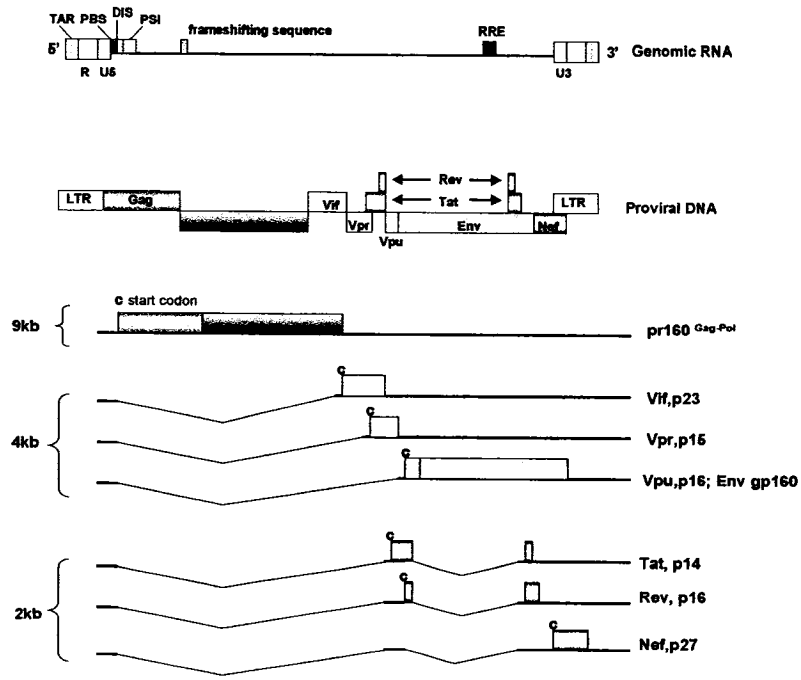
1.2 Organization and structure of the virus

An overview of the general structure of this virus; the genome, the viral protein and the characteristics of the virion will be described in this section.

1.2.1 Genome and Proteins

The HIV-1 genome is a 9 kb RNA flanked by two long terminal repeats (LTR) transcribed into cDNA and then integrated into the host genome. Encoding for 15 proteins obtained from nine different open reading frames, the transcript required presence of different elements to ensure the integrity of the viral replication processes. The transactivation response element (TAR), the primer-binding site (PBS), the dimer initiation site (DIS), the packaging signal ψ (psi), the frameshifting sequence and the Rev-responsive element (RRE) are all cis-acting sequences necessary in different steps of the viral cycle. Other elements are also known to be involved in post-transcriptional regulation of the RNA, such as the splice acceptor site and splice donor site. These ones are described to participate in the regulation process of different cleavage sites of the different messenger RNA (mRNA), the species obtained by alternative splicing of the full length RNA will encode the different viral proteins [31] (Figure 1.1A).

A. HIV-1 Transcripts



B. HIV-1 Proteins

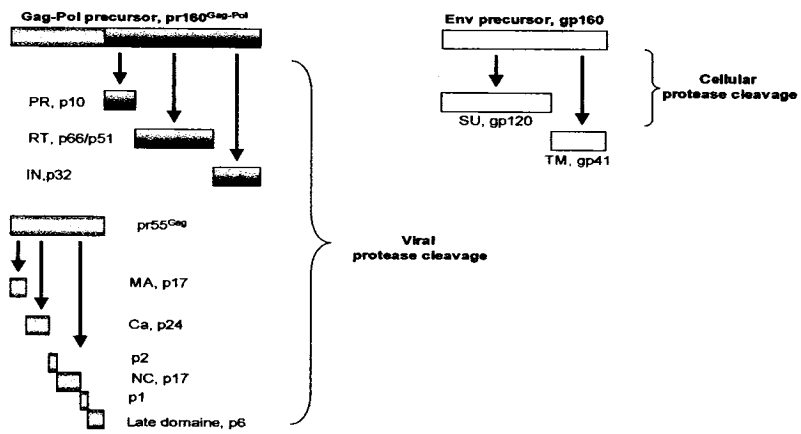


Figure 1.1 Organization of the HIV-1 Genome . A. HIV-1 Transcript. Key elements that are important for different steps in the viral replication cycle, are represented in colour. Following the integration in the host, the 9kb viral genome contains 3 open reading frames encoding for 15 proteins, which will be transcribed and then spliced in more than 30 different mRNA species. Above, initiation codons are indicated. **B.** HIV-1 proteins. Viral protease produces process the $pr55^{Gag}$ and the $pr160^{Gag\text{-}Pol}$ polyproteins into different subunits. Env is cleaved by a furin-like cellular protease that is found present in the Golgi, into two parts. Adapted from Peterlin et al.[32]

1.2.1.1 Viral spliced RNA

Several different mRNAs are formed from the splicing of the full length 9kb RNA. These mRNAs will produce the group specific antigen (Gag) and the Gag-Pol polyprotein. Two other groups are observed: the 4kb that are single-spliced encodes for viral infectivity factor (Vif), Vpr, viral protein U (Vpu) and Env gp160. The fully spliced encodes for the proteins transcriptional transactivator (Tat), regulator for gene expression (Rev) and negative regulatory factor (Nef) [31] (Figure 1.1A).

1.2.1.2 Structural and enzymatic proteins

$Pr55^{Gag}$, a precursor polyprotein of 55 kDa, is encoded by the Gag gene, which will be cleaved by the viral protease (Figure 1.1B). During the translation of Gag mRNA, a rare frameshift of -1 can occur. A specific sequence in the genomic RNA will allow the ribosome to slip in a -1 frameshift where it will bypass the stop codon and allow next tRNA to be positioned and to continue the protein elongation of $pr55^{Gag}$ and to produce $pr160^{Gag\text{-}Pol}$, precursor of the viral enzymes. To ensure the integrity of the virus, a ratio of 20:1 of $pr55^{Gag}/pr160^{Gag\text{-}Pol}$ is always favored [33]. Throughout the replication cycle different enzymes will be required, which will be generated through the cleavage of $pr160^{Gag\text{-}Pol}$ into different segments: PR, RT and the integrase (IN). The envelope glycoprotein Env is produced by cleavage of the Env precursor by a cellular furin protein in the Golgi. This event will allow the formation of the surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41) followed by their transport to the cell membrane[31] .

1.2.1.3 Regulatory and accessory proteins

HIV-1 is part of the complex retrovirus group, in contrast of the simple retrovirus, it encodes different proteins known as regulatory proteins and the accessory proteins. Even if these proteins are not essential *in vitro*, they are important *in vivo* at different steps in the viral replication cycle and the progression of the AIDS disease. Tat, Rev and Nef are the regulatory proteins and Vif, Vpr and Vpu are the accessory proteins [34] (Figure1.1B). Role of these proteins will be described later.

1.2.2 The virion

HIV-1 is an enveloped virus surrounded by a bilipidic membrane which is obtained through viral budding from the host cell. Through this budding process there is intercalation of different cellular molecules and proteins, such as sphingomyelin and cholesterol, as well as the glycoprotein Env. With a composition of 1-2% of RNA, 35% of lipids and 65% of proteins, the viral particles are characterized by a stable dimer of two positive-sense single-stranded genomic RNAs. Furthermore, approximately 1% of the produced virus particles are infectious. The virion has to pass through a maturation step allowing the immature particles and the Gag proteins, to acquire a functional morphology for future infection. The immature and the mature forms are similar for most of the part in general content. However, Gag processing will affect integrity of the protein content by generating sub-proteins product from the cleavage. All other viral proteins such as RT, IN, the PR, Vpr, Vif, Nef and even Env ,which will be cleaved before its encapsidation and will not be influenced really by the maturation process. In fact, the precursor of Env proteins are cleaved in the cell allowing formation of a trimer of the different sub-domain the SU (gp120) at the external part of the lipids envelope and the TM spanning the lipids envelope [23, 31].

1.2.2.1 Immature virion

After the budding process, the viral particles are immature and potentially non infectious and are observed mostly as a sphere surrounded by an electron-dense ring structure composed of multimerized $pr55^{Gag}$ (Figure 1.2A). Described to be relatively stable in weak detergent compared to the mature form, it may be explained the structural demands of the different steps leading to the virus assembly which require much more, in contrast of the disassembly process which where the virus has to be more flexible [23, 35].

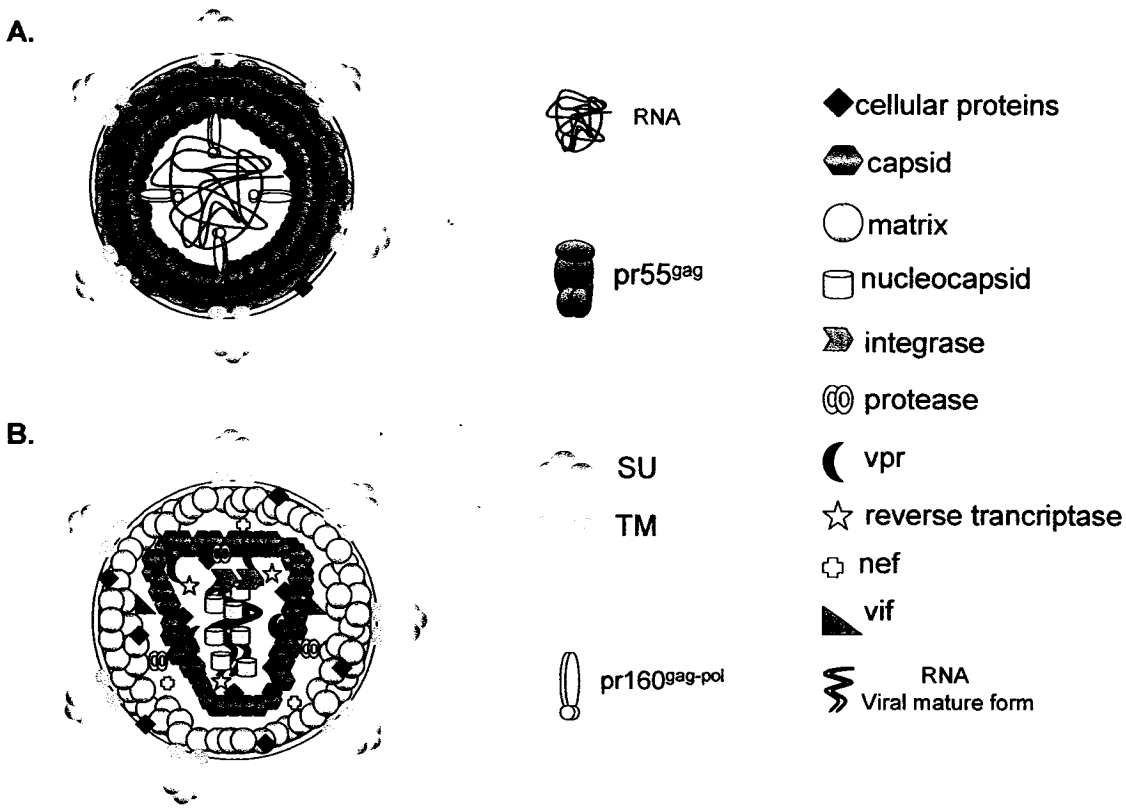


Figure 1.2 HIV-1 Virion . A. Immature virion. Schematic representation of an immature HIV-1 particle. Approximate position of the major proteins and the RNA are indicated. Details are provided in the text. Adapted from Retrovirus [23]. **B.** Mature virion. Schematic representation of a mature HIV-1 particle. Approximate position of the major proteins and the RNA are indicated. Details are provided in the text. Adapted from Peterlin et al. [32]

Table 1.1 Host cellular proteins by HIV-1

Host molecules	Cellular function	Incorporated	Reference
CD45	Membrane phosphatse	no	37
CD46	Complement control protein	yes	38,39
CD55	Complement control protein	yes	39,40
CD59	Complement control protein	yes	39,40
Actin	Cytosqueletal protein	yes	41
Pin 1	Peptidyl-prolyl isomerase	yes	51
tRNA synthetase	Ligase	yes	42
GAPDH	Aldehyde oxidoreductase	yes	43
MAPK ERK2	Serine/threonine kinase	yes	44
HSP27	HSPs chaperone	no	45
HSP60	HSPs chaperone	no	45
HSP70	HSPs chaperone	yes	45
HSC70	HSPs chaperone	yes	45
HSP90	HSPs chaperone	yes	45
CypA	Immunophilin	yes	46
CypB	Immunophilin	no	46
FKBP12	Protein degradation and sorting	yes	47
Ubiquitin	Vesicular transport protein	yes	43
Tgs101	Vesicular transport protein	yes	48
Tal	Vesicular transport protein	yes	49
VPS28	Vesicular transport protein	yes	50
AIP1	Vesicular transport protein	yes	50
VPS4B	Vesicular transport protein	yes	50
VPS37B	Vesicular transport protein	no	51
APOBEC3G	Cytosine deaminase	yes	52
APOBEC3F	Cytosine deaminase	yes	53
UNG	Uracyl-DNA glycosylase	yes	56
Staufen	RNA binding protein	yes	55
HLA-DR	Antigen presentation	yes	57
ICAM-1	Adhesion molecule	yes	58
Ezrin/myoesin/cofilin	Cytoskeletal proteins	yes	44
Thy-1	GP1-anchored protein	yes	37
GM1	Ganglioside	yes	59
Other cell surface constituents			57, 36

Adapted from Cantin et al. [36]

1.2.2.2 Mature Virion

To acquire a mature form, the multimerized Gag precursors have to be cleaved by the viral proteases in order to create potential infectious particles. This step involves a conformational and structural rearrangement of the core where the spherical immature virion will convert to a conical shell form and become a mature virion (Figure 1.2B). This maturation process leads to the production of around 2000 proteins of capsid (CA) that will form the condensed shell core, the matrix (MA) is surrounding the core by the inner face, while nucleocapsid (NC) surround the dimer of genomic RNA, whereas the location of the p6 protein is not really known. The susceptibility of the mature virions to detergent may be explained by a less-clustered capside protein arrangement, which may facilitate the dissociation step of the replication process [23, 35, 60].

1.3 Replication cycle of HIV-1 and implication of cellular proteins

The viral replication cycle is complex and involves cooperation of different viral and cellular proteins. Different reasons can be raised for the need of host proteins in the process, but these ones are essential and critical; viruses are incapable to replicate by themselves and need to develop alternative approaches to take advantage of cellular processes for their benefit. The replication cycle of HIV-1 can be divided in different stages and sub-classified either as the early phase, which represent the steps prior the incorporation of the viral genomic material in the host genome, or the late step, which follows the integration and leads to the formation of viral particles [31]. In this section, an overview of those different inter-related steps will be presented and linked to the virus-host interactions and different cellular proteins will be presented [30, 61] (Table 1.2).

Table 1.2 Host cellular factors involed in HIV-1 replication cycle

Host factor	Descritpion	Role in HIV-1 replication
CD4	55 kDa member of the immunoglobulin	Primary HIV-1 receptor; promotes binding and fusion through interaction
Coreceptors (CXCR4, CCR5)	G-protein-coupled chemokine receptors	Interact with gp120 following CD4 binding to promote membrane fusion
Lipid rafts	Lipid microdomains rich in cholesterol and glycosphingolipids	Promote virus entry and assembly/release
DC-SIGN	C-type lectin	Expressed on dendritic cells; capture HIV-1 virion and stimulates infection of CD4+/coreceptor+ cell in trans
APOBEC3G	Cytidine deaminase	Induces G to C hypermutation during reverse transcription; its activity is counteracted by the viral protein Vif
CypA	Peptidyl-prolyl cis-trans isomerase	Interact with CA during assembly and perhaps post-entry to counter antiviral restriction
Refl	unknown	Restricts some retroviral infections in human cells
LV1	unknown	Restricts lentiviral infection in some non-human primate cells
BAF	Double-stranded DNA bridging protein	Component of retroviral PIC; stimulates retroviral integration; inhibits autointegration
Ini1	Component of SNF-SWI chromatin remodeling complex	Binds IN; stimulates integration
HMGa1	High mobility group DNA-binding protein	Component of HIV-1 PIC; stimulates retroviral integration
LEDGF/p75	Transcriptional co-activator	Binds IN; might stimulate HIV-1 IN nuclear import and integration
P-TEF-b	Complex composed of CDK9 and cyclin T	In conjunction with Tat, promotes transcriptional elongation
CRM 1	Nuclear export factor	Promotes Rev-mediated nuclear export of unspliced or partially spliced HIV-1 RNAs in conjunction with the Ran GTPase
HP68	RNaseL inhibitor	Promotes viral assembly
ESCRT-I	Cellular endosomal sorting machinery	ESCRT-1 component Tsg101 interacts directly with HIV-1 p6; sorting machinery promotes virus release from the cell surface
ESCRT-II		
ESCRT-III		
AIP1		
AAA ATPase Vps 4		

From Freed [62]

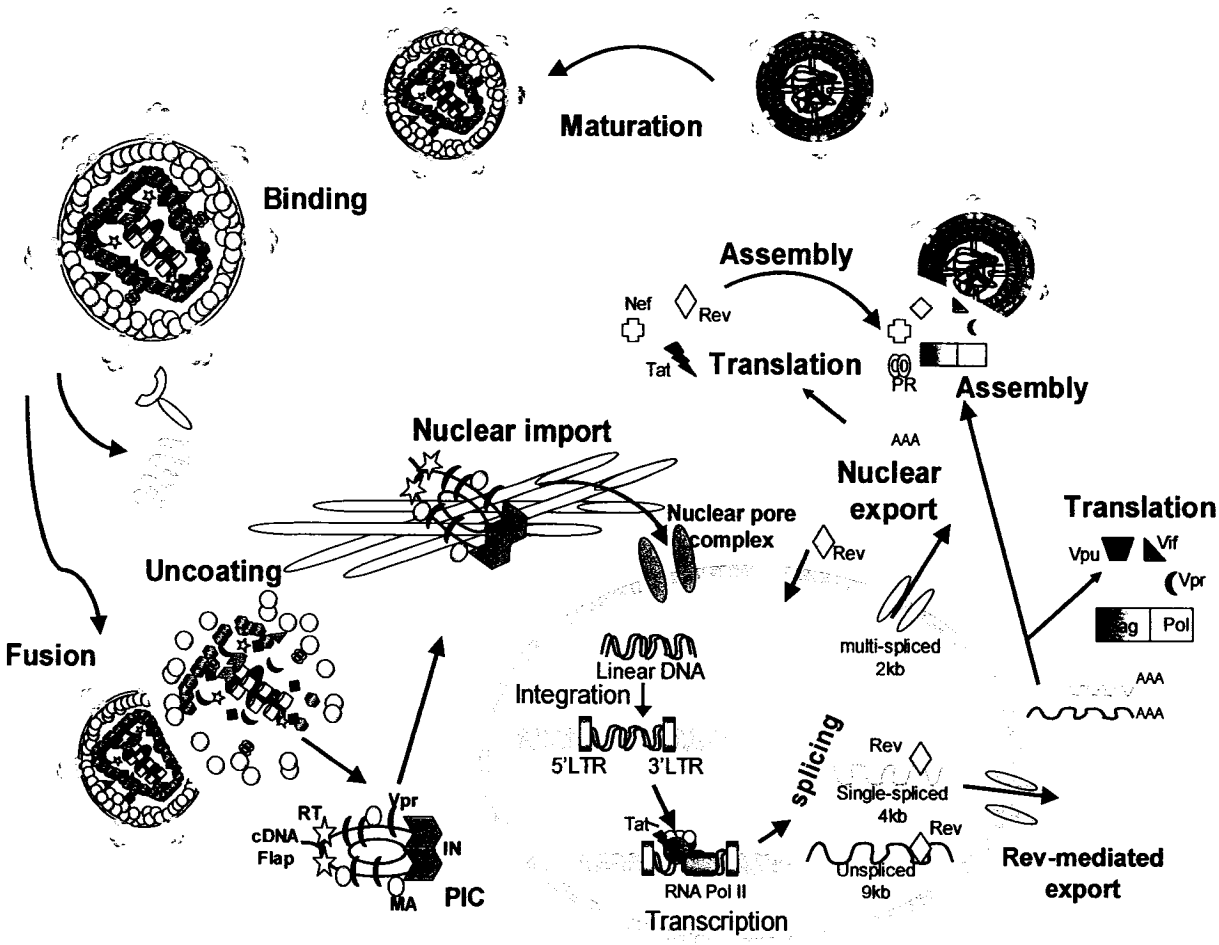


Figure 1.3 HIV-1 Replication Cycle. The virus enters the cell via cell membrane fusion of gp-120 trimer, the CD4 receptor, and the chemokine receptors, CCR5 or CXCR4. Capsid will be then uncoated, reverse transcribed and simultaneously formed the PIC with protein such as IN, MA and Vpr and cellular proteins to be imported into the nucleus. In the nucleus, it will integrate into the host genome and the transcription can start at the 5'LTR. Once produce Tat will increase LTR activity which increase the viral replication activity. Rev shuttles between the nucleus and the cytoplasm which will help to export the unspliced and the single-spliced RNA transcript to allow expression of the structural and the enzymatic proteins once in the cytoplasm. The $pr55^{Gag}$ will recruit different cellular factors which will participate in different way in the late step such as the assembly, the RNA encapsidation and the budding. See the text for more information. Adapted from Peterlin et al.[32]

1.3.1 The early steps

1.3.1.1 Virus entry

To infect the host, HIV-1 enters the cell by viral-cell membrane fusion which involves a high affinity binding of the glycoprotein gp-120 trimer, located on the virion, and the CD4 receptor, of the future host cell, generating an important change of conformation that will allow exposure to the co-receptor. Depending of the sub-domain present on the cell envelope, either the β-chemokine CCR5, which are the receptor of macrophages and dentritic cell, or CXCR4, the α-chemokine receptor of T lymphoctes, will be used as co-receptor to support the membrane fusion and allowing released of the viral core into the cell cytoplasm [31, 63]. Other molecules have been suggested to capture HIV-1 in non-lymphocyte cells. The dentritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) has been described to capture the virus, however this molecules cannot induce virus-cell fusion[64]. The inclusion of different cellular proteins on the viral particles such as the human leukocyte antigen II (HLA II) and the intercellular adhesion molecules type 1 (ICAM-1) may increase the potential interaction between the viral envelope and the host membrane.

1.3.1.2 Decapsidation

Several findings have shown that different viral proteins, such Nef and Vif, and other cellular proteins were required for the decapsidation process. However the sensitivity of the viral cores to the isolation technique using detergent raised several questions and lead to the development of other techniques to keep the cores stable [65, 66]. In the cytoplasm, the viral core undergoes a disassembly process which involves the MA phosphorylation by mitogen-activated protein kinases (MAPK). This favors the cell membrane detachment and the formation of the pre-integration complex and its nuclear translocation [67-69]. It has been proposed that phosphorylation of the CA protein may interact with

the cellular protein cyclophilin A, helping in the dissociation of the CA dimer [70, 71]. In contrast to the cellular protagonist proteins in the cycle, the cellular tripartite interaction motif 5α (TRIM5α) protein was shown to have an inhibitory effect through potential interaction of its carboxy-terminal domain with the CA protein [72, 73].

1.3.1.3 Reverse transcription and nuclear import

Formation of the reverse transcriptase complex (RTC) involves disassembly of the core in a way that the MA, NC, RT, IN, VPR and the RNA remain associated allowing formation of the scaffold for the conversion of the viral genomic RNA in cDNA by the RT. To generate the cDNA, a tRNA primer binds the primer binding (PBS) site of the genomic RNA and allows the RT to start simultaneously the synthesis of the RNA/DNA hybrids and, degradation of the RNA, to avoid interference, by the RNAse H domain from the RT. Two DNA strand-transfer will occur during the process [23, 31]. The first jump allows the newly synthesized transcripts to reach a homology region, the polypurine tract (PPT), and allows reverse transcription of the first strand. The second jump will allow transcription of the second strand and formation of the DNA flap[74], a potential major element of the PIC complex, whose is presence is still subject to debate [75]. Different treatments are trying to target this step to eventually prevent the formation of new viruses by interrupting the transmission of viral genetic information. Examples of these treatments are nucleoside reverse transcriptase inhibitor (NRTI), which causes a premature termination of the newly synthesized transcript, and the non-nucleotide analogue (NNRTI), which will interfere with the catalytic site of the RT [23].

The immune system uses different strategies to prevent genetic viral transmission and one of them involves the cellular protein APOBEC3G. This cellular protein is a family member of a group which will act on the RNA/DNA, allowing deamination of the cytosine causing hypermutation in the viral genome and degradation of the This cellular

protein is a family member of a group which will act on the RNA/DNA, allowing deamination of the cytosine causing hypermutation in the viral genome and degradation of the cDNA in process of being formed. To avoid this degradation, the viral protein Vif can interact with APOBEC3G, causing ubiquitinylation followed by proteosomal degradation [52].

Different viral proteins, such MA, IN and Vpr, seem to be essential for importation of PIC into the nucleus and subsequent steps. In fact MA and Vpr contain a specific nuclear localization signal leading to nuclear import [67, 76, 77]. However few arguments have been proposed that those proteins are not essential and are more related to the CA. This is because the virus can integrate itself into the host genome even if the provirus is depleted of those proteins and that presence of Vpr, MA and IN to the MLV genome do not lead to integration. Differences in the CA protein may explain the differences between HIV-1 and MLV, as well as the potential effect of the MLV CA to hide the NLS signal [78].

To get into the nucleus, cDNA compaction by the high mobility group A (HMGA1) is required and involves the formation of an active integration complex [79]. This complex will target nondividing cells by inducing cell cycle arrest in G2 phase by Vpr inhibition on the cyclin-dependent kinase $p34^{cdc2}$ [80]. Different cellular proteins, such as the virion-associated matrix-interacting protein (VAN), have been suggested to participate in the potential nuclear import of the PIC complex by interacting with MA [81].

1.3.1.4 Integration

It is still unclear by which mechanisms cDNA initiates integration in the host genome, but once it gets into the nucleus generation of viral RNA transcripts occurs as if they were cellular gene transcripts. Viral cDNA integration does not have specific location preference, yet there is evidence that it may incorporate itself in highly active transcriptional regions to facilitate the production of its genome [82] and even some evidence suggesting that it targets certain "hotspot" regions [83, 84].

The process of integration starts by IN cuts to the 3' of the viral cDNA, which produces a 3'-recessed end on each extremity, and the host genome DNA. This enables insertion of the viral fragment into the host cell genome. Different cellular enzymes, such the Ku70, Ku80 factor [85] and ATM [86], will then participate in the non-homologous DNA-end joining, allowing fusion of the viral/host genomes [31].

1.3.2 The late steps

1.3.2.1 Gene transcription

Presence of two long terminal repeats (LTR) is important and have been described to direct transcription. Located at each end of the provirus, they can be divided in three different regions: the U3 (for unique 3' end), the R (for the repeat region) and the U5 (for the unique 5'end). U3/R region is well known to contain different elements such as a TATA box. The TATA box is essential in the recruitment of the cellular transcription factor IID (TFIID) and the cellular proteins sp1 and NF-kB, which allows the formation of the transcription complex and the positioning of the RNA polymerase II at the initiation site [31, 87, 88]. Different cellular proteins such as Murr1 will act and inhibit the proteosomal degradation of IkB, allowing an accute presence of IkB. The increase in IkB will lead to retention of NF-kB in the cytoplasm by the IkB-NF-kB interaction [89]. This retention will result in nuclear depletion of NF-kB, thus affect the transcription rate.

Transcription is initiated by the LTR. LTR has a low transcription rate which will be rescued by Tat. Once Tat has been produced, it will transactivate the LTR by a direct interaction with the transactivation response element (TAR). This interaction will increase the transcription rate by two logs [90]. Tat accomplishes this by interacting with the cellular cyclin T1, which will recruit the cellular cyclin kinase-dependant family (CDK9). Recruitment of the cyclin T1 and CDK9 to the TAR region will also recruit the necessary factors to phosphorylate the C-terminal domain (CTD) of the RNA polymerase

II, which will then activate the transcription [91]. Moreover, interaction between Vpr and the coactivator complex p300/CBP suggests an important role in LTR induction [92].

1.3.2.2 Viral RNA transcript: from the nucleus to the cytoplasm

Splicing produces over 30 different transcripts grouped in one of the 2, 4 or 9 kb RNA transcript categories [93], each encoding for different viral proteins. When the fully spliced 2 kb RNA is produced it will be exported out of the nucleus where its translation will be achieved A number of important transcripts are produced: 1) Tat protein, which increases the transcription rate [90], 2) Rev protein, which has a critical role in RNA transcript export, and 3) Nef protein, which induces downregulation of CD4 and major histocompatibility complex I (MHC I) [94]. Sequestered in the nucleus, the single spliced RNA 4kb and the unspliced RNA 9kb need the presence of Rev to be exported into the cytoplasm. Once Rev is translated and translocated to the nucleus and reaches the required nucleoplasmic concentration of Rev, it will multimerize and will interact with a specific RNA sequence of 250 nt, the Rev-responsive element (RRE). Present in the 4 kb and the 9kb transcripts, this highly structured element interacts with Rev allowing formation of a complex which will then interact with the cellular export machinery, CRM1, a receptor for nuclear export of components such as exportin and RanGTP. This interaction will collaborate with the RNA export of the 4 kb and the 9 kb out of the nucleus [95], then translation and expression of the different structural proteins. Presence of cellular proteins human Rev-interacting protein (hRip) / Ras-associated small GTPases (Rab) and Nup98 have a significant impact on Rev action by formation of potential bridges between Rev and CRM1, allowing RNA transport and it subsequent release in the cytoplasm and the perinuclear region [96-98]. Sam68, a cellular RNA helicase A which has been suggested to be involved in post-transcriptional regulation of HIV-1, may interact with Rev to ensure RNA integrity and complex stabilization. Recently, the DEAD box protein RNA helicases, DDX1 and DDX3, have been shown to bind the nuclear export protein CRM1, which is essential for nuclear export mediated by the HIV-1 Rev protein, shuttles between the cytoplasm and nucleus, facilitates the nuclear export

of RRE-containing RNA in the presence of Rev [99]. Moreover, other cellular proteins such the eukaryotic initiation factor 5A (eIF5A) and the heterogeneous nuclear ribonucleoproteins (hnRNPs) play different roles in RNA export, metabolism and translation regulation [100].

It has been suggested that formation of ribonucleoprotein (RNP) complex in the cytoplasm occurs simultaneously with the disassembly of the export complex. Different RNA binding proteins are involved in the transport of the RNA. To contribute to this process, they may heterodimerize together in a complex and form a RNP complex which may help the transport along the microtubule. Each RNP complex contains motor molecules, such as dynein and kinesin, which will move toward either end of the microtubules. The motor molecules can go towards the minus end of microtubules, which is known as the retrograde route, or towards the plus end, which is known as the anterograde route [101]. The composition of RNP complexes plays an important role in determining the route taken on the microtubules, via the interaction with specific motor molecules. Formation of those RNP complexes may stabilize the transcript until its translation by the cellular machinery.

The translation process allows generation of the regulatory, accessory and structural proteins which are required in the viral cycle. To promote viral translation over the cellular translation, cooperation between viral and cellular proteins at different levels will modulate the switch. One example is the TAR RNA-binding protein (TRBP), which stimulates the translation of the TAR-containing RNAs by binding PKR (dsRNA-dependent serine/threonine protein kinase) [102]. Once the PKR-TRBP complex is formed, the interaction in the complex alters kinase function of PKR and inhibits phosphorylation of the eukaryotic initiation factor eIF2alpha, which is an essential cellular protein for synthesis [102]. In fact, eIF2 is an oligomer composed of three subunits which will interact with GTP and the initiator methionyl-tRNA (Met-tRNAi) to form a ternary complex that will join intiator tRNA to the 43s preinitiation complex.

However, phosphorylation of eIF2 inhibits the subsequent step leading to the formation of the translation initiation complex which also promotes the accumulation of unstranslated mRNA [103]. Since the virus uses the cellular translation system, therefore it wants to maximize translation of the viral protein over the cellular protein translation. Tat, a viral protein, will act as a substrate homologue for PKR and will, in this case, compete with eIF2alpha to decrease the cellular translation over the viral translation [103, 104].

1.3.2.3 Viral assembly

Expression of $pr55^{Gag}$ allows the formation of virus like particles (VLP), since it is a precursor of different structural proteins [105, 106]. Moreover, it contains major domains which target and bind the plasma membrane of the cell. Myristic acid present in the M domain of the MA part and the presence of highly basic domain in the 17 to 31 a.a. portion of $pr55^{Gag}$ and which are positively charge elements of $pr55^{Gag}$ and $pr160^{Gag\text{-}Pol}$ will target the cellular membrane [31, 107, 108] and favor accumulation in a specific region called lipids rafts which are rich in cholesterol [37, 109]. Env gp160 is transported to the plasma membrane through the Golgi, whereas $pr55^{Gag}$ and $pr160^{Gag\text{-}Pol}$ are transported to the cellular membrane by a microtubule dependant process. KIF-4 is a cellular motor protein of the kinesin superfamily which interacts with unprocessed $pr55^{Gag}$ and may act as a bridge between gag and the microtubule during transport to the cell membrane [110, 111].

During its transport to the cell membrane, the Gag interacts with different components. Recently, it has been suggested that the MA domain of $pr55^{Gag}$ [105] could interact with Env to allow recruitment of Env to plasma membrane, and also the presence of mutations in MA may cause abnormalities in Env incorporation [112, 113]. Formation of the virion at the plasma membrane involves gag-gag interaction and oligomerization. Several domains may be involved in the process, but the CA domain [114] and even more the interaction (I) domain [115], located in the nucleocapsid part, are the most important

ones. Those domains are described to favor gag-gag interaction and presence of mutations in those domains impairs with the assembly process [116, 117]. As the process of CA assembly requires ATP [118], yet $pr55^{Gag}$ does not bind to ATP itself, so another element has to be involved. The cellular protein HP68, a RNase L inhibitor, has been shown to bind ATP and to be involved in post-translational events of HIV-1 assembly. Moreover, it interacts with Vif through its Gag complex which will be involved in virion morphogenesis and infectivity [119].

Moreover, viral assembly requires trimerization of the Env. To be fully functional and to acquire its active form, Env has to go through a maturation process which involves glycosylation of the gp120 portion, formation of disulfide bonds on the gp160 part, and finally cleavage of $pr160^{Gag\text{-}Pol}$ into the SU domain (gp120) and the TM domain (gp41) by furin-like enzyme found in the Golgi during Env trafficking to the membrane [31]. To help the trafficking process of Env to the cell membrane, Vpu will bind to CD4 and targets it for proteolysis by the cytosolic ubiquitin-proteasome pathway, which will downregulate the CD4 receptor and consequently lead to the Env release from the CD4/Env complex allowing Env transport to the cell surface [31, 60, 120, 121]. Additionally, Nef is also described to play an important role in CD4 degradation, which will also promote transport of Env to the cell membrane. In fact, Nef will link to the CD4 localized at the cell surface to clathrin-coated pits via interaction with the dileucine sorting motif present in the cytolasmic tail of CD4, which depend on an adaptor protein complex mechanism (AP), resulting in degradation by the lysosomes [122, 123]

Finally, one of the major components in viral assembly and in virus formation is the cytoskeleton network. Different cellular molecules are related to this network, such as the actin filament, the microtubules and their associated binding proteins which are involved in different cellular functions, like cell polarity, but also in the cytoplasmic trafficking phenomena[124]. Moreover, viral particles production seem to be controlled by the cytoskeleton as well [125, 126] and may act as a route to direct all the viral components to their destination.

1.3.2.4 Viral RNA incorporation

Steps leading to the genomic RNA incorporation are still unclear and are subject of debate. Few hypotheses explain that the 9kb transcript is introduced in the viral particle only when the virion will be formed at the plasma membrane. On the other hand, several groups believe that HIV-1 may prefer to encapsidate the genomic RNA that has just been translated by the ribosome [127, 128]. However, there are others who think that two different pools of RNA may exist: one that will be encapsidated and another that will be translated. Even if this step remains unclear, specific encapsidation of two copies of 9 kb genomic RNA transcripts are mediated by the necessary interaction of the NC domain of $pr55^{Gag}$ with ψ, present only in the genomic RNA [31, 129]. RNA dimerisation occurs in a specific region described as the dimer initiation signal (DIS), present near the 5' end of genomic RNA.

Moreover, different proteins are known to influence RNA encapsidation. In fact, Staufen, a double-stranded RNA (dsRNA)-binding protein, is incorporated in human immunodeficiency virus type 1 (HIV-1) and is suggested to be implicated in retroviral genome selection and packaging into assembling virions [55, 130].

1.3.2.5 Budding

Described as a second membrane fusion, the viral budding involves the p6 domain, a critical domain of $pr55^{Gag}$. Also named L domain, for its role in the late step of assembly, p6 is known to interact with the cellular ubiquitinylation and endosomal sorting process. Mutations in this domain, which appear mostly in a specific region in the C-terminal called the PTAP motif, lead to the formation and release of defective viral particles [131, 132]. Recently, detailed analysis of this motif revealed that mono- ubiquitinylation of this sequence by a ubiquitin-protein ligase hPOSH (human plenty of SH3s) was essential to

the release of the newly synthesized virus [133]. Several studies have identified this conserved domain as an important sequence interacting with cellular proteins such as Tsg101 [48]. Described as a member of the class E vacuolar protein sorting system (Vps) and structurally related to the yeast protein Vps23, involved in the vacuole sorting pathway, Tsg101 is known to be part of the ESCRT-1 complex (endosomal sorting complexes required for transport) [134]. In the case of HIV-1, Gag recruits Tsg101, which will then allow recruitment of ESCRT-I, the AP-1 complex, and finally ESCRT-III [135]. Recruitment of these complexes, well known to be involved in the vesicular sorting system, is subject to debate because it is believed that the assembly and budding takes place at the cell membrane. However it is well know that the virus present in the macrophages are using multivesicular bodies (MVB) and the vesicular sorting pathway for viral budding [48, 136]. Three models are suggested for HIV-1 budding which are influenced by the cell type: the lipid raft budding model, the Trojan exosome budding hypothesis which involve the MBV and the cell to cell contact.

In the first model, multimerised Gag-Gag proteins are directed to the lipid raft which is enriched of sphingolipids, cholesterol and glycoprotein-linked lipids and is detergent resistant. It has been demonstrated that HIV-1 may prefer those domains to complete the viral assembly and then be release form the membrane [37]. On another hand, it has been suggested that HIV-1 will hijack the pre-existing exosomal exchange pathway for its own propagation based on MVB. The viral complex will be transported to the cell surface and released by fusion of the MVB with the cell membrane [37, 137]. Finally, the cell to cell system is a system involving direct recruitment of different receptors necessary in the fusion process of cell infection. The virus is directed to another cell by a direct contact between the infected cell and the target cell. [138].

1.3.2.6 Maturation

The newly synthesized viral particles are immature and require maturation process which allows a series of structural rearrangements from a doughnut shape to an electron-dense shaped conical core. This process will be crucial for the viral infectivity and the reinfection process. To achieve this process, the viral protease (PR) forms a homodimer to acquire full activity following auto cleavage [139]. Once activated, PR will cleave, in an ordered process, the $pr55^{Gag}$ present in the nascent virion. Different studies have shown that the first cleavage is at the C-terminus of p2, which will release the RNA-binding NC protein and will lead to the condensation of the RNP core. The next step is the separation of MA and CA, which will alter the stability of the CA-CA structure. The final step of maturation, in which the final condensation of the capsid will occur, requires the cleavage of SP1 from the C terminus of CA [140, 141]. Due to its essential role in the replication cycle, PR is one of the targets during HIV-1 therapies.

1.4. RNA trafficking

Many studies tried to understand the different steps in the viral replication cycle. Even with these studies, several steps remain unresolved. The subject of my MSc project primarily tries to elucidate the steps following the genomic RNA nuclear export, which involves the RNA trafficking in the cytoplasm, and the subsequent steps leading to the assembly of the virus.

For a few years, several research proposals tried to characterize different pathways involving viral and cellular RNA trafficking. Here, a brief overview about RNA trafficking will be presented.

1.4.1 General concepts about cellular mRNA trafficking

Proteins are not spread equally in the cell. Sometime a protein can be required in one part of the cell and yet, at the same time, can be harmful in another part. The mechanism responsible for maintenance of this polarity, involving a close association with different elements relating to the microtubules, requires cooperation of different steps in translation and RNA stability [124, 142]. Several proteins do not encode for a sorting signal which targets protein to their specific site. Instead, to ensure their distribution, the cell will use mRNA localization as a means of targeted protein distribution. This will prevent protein expression which could be harmful to the cell and therefore force the cell to restrict the transcription to individual regions in the cytoplasm and enable the rapid response to local requirements [143].

1.4.1.1 Mechanism of RNA localization

To ensure specificity, a mRNA *cis*-acting sequence, present principally in the 3' untranslated region (UTR) part of the transcript, will be required, in combination with a *trans*-acting factor which will bind the signal, to transport the transcript at its specific site [124]. However, the transport signal is not restricted specifically to this region and may be found as well in the 5'UTR or elsewhere [144, 145]. Moreover, the presence in mRNA of the *cis*-acting sequence, which allows binding of a *trans*-acting factor, will form a complex, also known as a ribonucleoprotein, RNP, complex, with other proteins involved in mRNA protection and localization to its final site of action.

Different mechanisms of RNA localization have now been described. Even though, mRNA is known to use a one type of mechanism, this does not exclude the use of another mode of trafficking [143]. The first type of mechanism is related to the local translation of the mRNA. This involves the trafficking of the transcript to a particular region and then its translation. A second type of mechanism describes the local protection of RNA

from degradation. It involves two different *cis*-acting elements: the first element targets the RNA for its degradation; the second element binds the RNA for its protection, which stabilizes the transcript. A third mode of trafficking involves diffusion and anchoring, in which mRNA can passively diffuse through the cytoplasm until it is trapped by a localized anchor. Finally, mRNA can also be trafficked to it site of action by active transport along the cytoskeleton network [143].

1.4.1.2 Example of mRNA localization in different organism

As mentioned earlier, the cytoskeleton is important in different steps and is necessary to keep the integrity of the cell. In *Saccharomyces cerevisiae*, localization of achaete-scute homologue 1 (ASH1) mRNA is actin dependent. In fact, a protein called She1 is known to transport ASH1 mRNA to the bud tip [146], which will result in repression of the mating-type switch in the daughter cell [147]. ASH1 mRNA is known to have four *cis*-acting elements which bind She1. This will result in formation of a complex involving proteins, such as She2 and She 3, which will cooperate together [148].

The most well known cases in mRNA localization is in *Drosophila melanogaster*. Oocyte development requires generation of an asymmetric distribution and polarity in the cell and different mRNA are known to be localized[149]. Different RNA binding proteins are known to interact with different RNA transcripts, allowing their trafficking to the translation site. The double-stranded RNA-binding protein (dsRBP) Staufen is known to bind the *cis*-acting sequence of *oskar*, found in the 3'UTR portion of the transcript. However it also requires the presence of other proteins, such as Margoh, which will recognize the exon-exon junction complex (EJC). The EJC is deposited on mRNAs in a splicing-dependent manner, 20–24 nucleotides upstream of exon–exon junctions, independently of the RNA sequence which will be assembled at splice junctions after splicing is completed, indicating a possible role of splicing in oskar mRNA localization. These interactions will then allow the complex to be trafficked to the posterior pole of the oocyte [150]. . Another well known family of proteins involved in RNA localization are

the heterogeneous nuclear ribonucleoprotein, hnRNPs. In Drosophila, Squid protein (Sqd) is known to bind *gurken* mRNA to allow its trafficking along the microtubules towards the dorsal pole of the oocyte, which leads to the formation of the perpendicular dorso-ventral axis [151].

Xenopus laevis is another system that is used to study RNA trafficking. This system is considered an important model which possesses several qualities, such as a larger nucleus than the somatic cells, faciliting RNA microinjection used in mRNA localization and transport studies. Two major mechanisms for mRNA localization were observed. The first one allows a protection/diffusion/entrapment mechanism in the early stage and the second one requires active localization in the later development stage [152]. Examples of the early stage mechanism are the *Xcat-2* and *Xlsirt* mRNAs, involved in germ-cell line migration and the acquisition of germ-line fate and localization to the vegetal pole. Due to the absence of the microtubule organizing center (MTOC) at this stage, RNA diffusion will form proteins aggregates, RNP complexes, at the message transport organizer region (METRO). Two different and non-redundant elements participate in Xcat-2 mRNA localization. The first one is known as a 240 nt element adjacent to the 3' end of the open reading frame, anchoring the RNA to the mitochondrial clouds (MC), described as a non-membrane space where mitochondrial replication and germ plasm, the hereditary material, formation takes place. The second element, which is located in the 3'UTR, is a 160 nt element, also described to be potentially involved in RNP complex formation, that binds a unknown trans-acting element [152, 153]. For its part, *Xlsirt* mRNA localization could be explained by formation of secondary structures, involved in RNA localization, defined as a varying numbers of 79-81 nt tandem repeats and one unique 137 nt sequence [154]. In the later stages, the cytoskeleton network is functional and the RNP complex may use it for RNA transport. Most RNA localization characterized in late stages involved the *Vg1* and *VegT* mRNAs, which are localized to the vegetal pole. Those two proteins are associated with VgRBP60/hnRNP I and Vg1RBP/Vera, suggesting that the RNA transport may start in the nucleus [155]. In fact, a localization signal of 340nt in *Vg1 RNA* and 300 nt in *VegT* have been identified in the 3'UTR [156, 157].

Finally, neural cells such as neurons and oligodendrocytes are systems widely used to study cell polarity and mRNA localization. Few mRNAs such as β-actin, TAU, MPB (myelin basic protein) and CaMKIIα (calcium/calmodulin-dependent kinase-2α) have been characterized and observed to localize in the axon. β-actin mRNA localization is related to the cytoskeleton. However, RNA trafficking varies depending on the cell type: for example, actin will be used in fibroblast and microtubules in the neurons. In each case, a 54 nt sequence, named Zipcode and described as an A-U rich region, will be involve in mRNA localization. *Trans*-acting proteins, such as the zipcode-binding protein (ZBD) 1 and ZBD-2 which are part of the hnRNP family, are already describe to help the process [158],[159]. HuD, a member of the RNP family of proteins, is also known to bind the U-rich elements, located in 3'UTR, and form a complex with microtubule [160]. Moreover, other mRNAs have been described to use kinesin and the microtubule network to be localized. In fact, MBP and CaMKIIα proteins are known to be involved in the myelinating process of neuronal cell [161]. However, to achieve their function they have to be localized. CaMKIIα mRNA localization might depend in part on the binding of the cytoplasmic polyadenylation element binding protein (CPEB) to the cytoplasmic polyadenylation element (CPE), founded in the 3'UTR. Different experiments of CPEB overexpression have demonstrated to increase the mRNA localization at their respectice site. In contrast, expression of a CEPB mutant, defective for interaction with molecular motors, inhibits the transport of the mRNA [162].

One of the clearest examples of mRNA localization is certainly MBP, which is mainly described in oligodendrocytes. Due to its strong interaction with the membrane, this protein can not be produced far from its site of action, because it would interact with the membrane along its journey. To avoid this, the cell will use RNA localization [124]. As with other studies, assays on MPB mRNA localization were done by microinjecting labeled mRNA into the perikaryon and then looking at the route used by the mRNA, using microscopy techniques such as confocal. A 21 nt element was identified to be responsible for RNA transport and localization of MBP mRNA: the RNA transport signal (RTS), that was later renamed hnRNP A2 response element (A2RE). Located in the 3'UTR of MPB mRNA, this cis-acting sequence is describe to bind proteins, but more

specifically hnRNP A2 [163]. The proposed mechanism for targeting this mRNA to the translation site involved the anterograde movement and the microtubule dependent route.

1.4.3 Viral RNA trafficking concepts

To successfully infect a host organism, the virus has to generate several copies of its genome to continue its replication and to infect other cells. To ensure that the genomic RNA produced will be used for insertion in viral particles, viruses have developed different pathways using viral and cellular regulatory proteins and cis-acting RNA elements which will selectively interact together. Certain retroviruses, as we saw earlier, use Rev which will bind the RRE and this will promote viral export. Additionally, certain retroviruses may depend also on the *cis*-acting constitutive transport RNA element (CTE), that has been shown to interact with the human TAP (hTAP) protein, promoting the export of the CTE-containing mRNAs from the nucleus of type D retroviruses [164]. Beside the nuclear export, cytoplasmic transport is also important in viral assembly. Viral pre-budding complexes are suggested to be routed on the endosome to the cell membrane. They proposed that Env and Gag will form a complex on the endosomal membrane or lysosomal membrane, which will then recruit genomic RNA as it is the case for MLV [165].

Moreover, hnRNP A2 response element, A2RE, has been shown to be involved in different trafficking pathways in the cell and it has also been demonstrated that viruses used this element to maximize the production of progeny virion. This presence of A2RE in several viruses (Table 1.3) suggests the importance of this element.

Table 1.3 A2RE-like sequences in Retroviral RNAs

Viruses	Genes	Position	A2RE-like sequences							
None	MBP	1380	GC	/CAA	/GGA	/CCA	/AAA	/GAA	/CCC	/U
HIV-1	*gag*	1192	GA	/CAA	/GGA	/CCA	/AAA	/GAA	/CCC	/U
HIV-2	*gag*	738	- A	/CAA	/GGA	/CCA	/AAA	/GAA	/CCC	/U
SIV	*gag*	586	GA	/CA -	/GGA	/CCA	/AAA	/GA -	/CC -	/U
FIV	*gag*	1474	GA	/CAA	/GGA	/- C -	/AA -	/GAA	/ - - -	/U
EIAV	*gag*	833	G -	/CAA	/GGA	/- C -	/AA -	/GAA	/CC -	/U
BIV	*gag*	1187	- -	/CA -	/GGA	/CC -	/AA -	/GA -	/CC -	/U
MPMV	*gag*	1613	- A	/CAA	/GGA	/CC -	/- A -	/GA -	/CC -	/U
HTLV-1	*gag*	1609	- -	/CAA	/GG -	/- C -	/- A -	/GA -	/CC -	/U
GLV	*gag*	1950	- -	/CA -	/GGA	/CC -	/- - A	/GAA	/CCC	/-
Mo-MuLV	*gag*	1429	- A	/CAA	/GG -	/CC -	/AA -	/GA -	/- C -	/-
BLV	*gag*	1364	- -	/CAA	/GG -	/CC -	/- - -	/GA -	/- - C	/U
RSV	*gag*	1566	- -	/CA -	/GGA	/CCA	/- - -	/GA -	/- CC	/U
ALV	*gag*	1790	- -	/CA -	/GGA	/CCA	/- - -	/GA -	/- CC	/U
FeLV	*gag*	1685	- -	/CAA	/GG -	/- - A		/GAA	/- C -	/-
Consensus			R	Q	G	P	K	E	P	
HIV-1	*vpr*	6157			/GGA	/GCC	/AUG	/AGA	/UCC	/U
HIV-2	*vpr*	5855			/- - -	/- - -	/AG -	/GAG	/- - C	/-
SIV	*vpr*	6174			/- - -	/U -A	/- G -	/- AU	/- - C	/-
Consensus			R	N	G	A	R	R	S	

From Mouland et al.[166]

1.4.3.1 hnRNP A2 response element (A2RE)

Described as a 21 nt element, A2RE was first known as the RNA transport sequence and is well known to be present in different mRNA transcripts and to participate with the intermediate of hnRNP A2 for the RNA localization phenomena. Disruption of this interaction could be abolished by the presence of mutation in a specific position of the A2RE element. Actually, a few specific point mutations, such as G6C, A8G, and G9A, have a great impact on the binding, whereas others, such as C2U and C3G, have little effect. Those mutational studies also revealed that the 11 first nucleotides where sufficient to bind hnRNP A2 protein and allow mRNA localization [101]. This sequence was also inserted into non-transported mRNA such *GFP* mRNA to demonstrate that this 21 nt sequence was sufficient to target mRNA for transport and succeeded in bringing the *GFP-A2RE* to the myelin compartment in oligodendrocytes [163].

As we saw earlier, A2RE is not restricted to one mRNA but can also be found in different mRNAs (Table 1.4). The interaction between A2RE and hnRNP A2 is very specific and is confirm with a dissociation constant (Kd) of 50nM [167], but other potential interactions, like with hnRNP A3 and a Kd of 276 nM, may also be observed [168]. In the case of hnRNP A1, even with a high homology of 68% of hnRNP A2 [169] and similarity with its RRM, interaction with the specific element A2RE is really low (Kd=10μM) [167].

1.4.4 HIV-1 and A2RE

A few years ago it was demonstrated that HIV-1 contains two A2RE-l RNA cis-acting sequences that may regulate the RNA transport of the viral transcript, in a hnRNP A2 dependent manner [166]. The viral genomic RNA contains two sequences, A2RE-1 and 2, while vif, vpr or tat-encoding RNAs only contain the A2RE-2 elements. A2RE-1 is located in the *gag* gene, from nt 1192 to nt 1213, which is also part of the major homology region (MHR) (Figure 1.4). It is found to be conserved across retroviruses and is known to be required in viral assembly, maturation, and infectivity [114]. The specific location of the A2RE-1 element in the MHR suggests an important role in the HIV-1 replication cycle. A2RE-2 is located in a region which overlaps between the 3'end of the *vpr* gene and the 5'end of the tat gene, in position nt 6157-6178, which is adjacent to another highly conserved region, the exon splicing element (ESSE2) of HIV-1 RNA [170, 171].

1.4.4.1 HIV-1 trafficking in oligodendrocytes

To confirm that A2RE is involved in the RNA trafficking of HIV-1, different experiments were performed. Localization of the intracellular HIV-1 RNAs is influenced by the presence of A2RE elements. To demonstrate this, oligodendrocytes were microinjected with wild type or mutated HIV-1 in a position A8G of the A2RE and then analysed. Wild type RNA was efficiently transported. However, the presence of a single point mutation, A8G, in the A2RE, caused a disruption in binding of hnRNP A2 to its element. This will abrogate transport of the A2RE-mutated RNAs and confined the RNAs to the cell perikaryon [166]. This observation suggests that the element is well conserved among different transcripts. The RNA trafficking function of A2RE is nucleotide sequence dependent and are sufficient to confer transport of RNA containing the A2RE sequence. HIV-1 RNA trafficking was found to be related to hnRNP A2 expression and selective binding; if the A2RE/hnRNP A2 binding is disrupted, the localization of A2RE-containg elements will be affected [166].

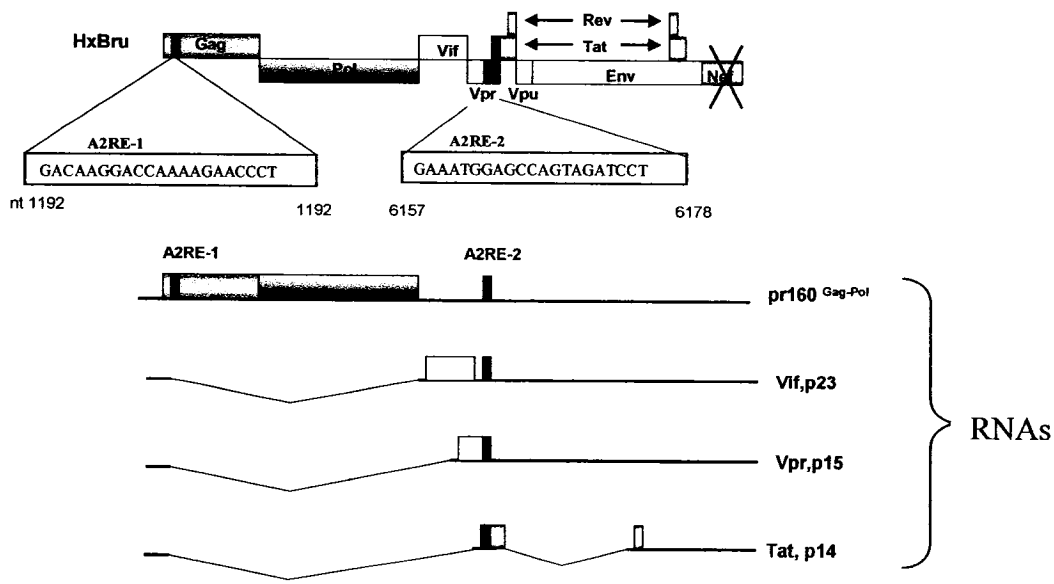


Figure 1.4 RNA Trafficking Signal in HIV-1. HIV-1 genomic RNA contains two A2RE cis-acting elements which are recognized by the cellular protein hnRNP A2 to promote the cytosolic RNA transport. The A2RE-1 is located in the MHR of CA and the A2RE-2 is located in an overlapping region between *vpr* and *tat* gene. Adapted from Bériault et al [172].

1.5 hnRNPs important cellular proteins: an overview of hnRNP A2 trafficking pathway

Following RNA transcription, the mRNA's fate relies on different mechanisms which ensure RNA stabilization of the transcript and its integrity. Posttranscriptional events, from the nucleus to the cytoplasm, are usually interconnected and several proteins are involved in this process. In fact, the RNA binding proteins called hnRNPs are likely to play a predominant and essential role. Until now, around 30 human proteins are associated with this family and known to form RNP particles with the nascent RNA transcripts [173]. hnRNPs are also described to regulate different cytoplasmic events such as RNA localization, which we described earlier, mRNA translation, and mRNA turnover [174]. In fact, the regulation pathway of gene expression in higher eukaryotic cells is post-transcriptional and involves processing of pre-mRNA, nucleocytoplasmic transport of this mRNA, and the localization in the cytoplasm. The group of hnRNP A/B is probably the most abundant protein of this family in the cell and seems to play a key role in the biogenesis and transport of mRNA [175]. Moreover, the fact that different studies have shown that mRNA transport, from the nucleus to the cytoplasm via the nuclear pore, is strongly associated with this group, which suggest an involvment in the export pathway of mRNA [176]. In other cases, like hnRNP U or other hnRNPs, there is a nuclear retention signal (NRS) which restricts the proteins to the nucleus. Recently it has been suggested that hnRNP U may be involved in an actin-based mechanism which is implicated in the transcription of most RNA Pol II genes. This protein may interact with actin and then carry out its regulatory role during the initial phases of transcription activation [177]. Due to their role in different cellular processes, different hnRNPs may have interconnected roles. Moreover, different domains may be shared between the members of this family, not restricted to one particular protein. Furthermore, it has been demonstrated that, due to the ressemblance of different hnRNPs, certain hnRNP may interact with a lower affinity with a specific response element of another hnRNP as we will describe later.

1.5.1 hnRNP A2

The sequence of human hnRNP A2 is relatively conserved and may have up to 95% homology with different species, such as the rat [175]. This 36kDa protein is produced from splicing of the 9kb *HNRNP A2B1* gene, located on the human chromosome 12. This gene also generates hnRNP B1, which is identical to A2, except it has an insertion of 12 a.a. near the N-terminus (a.a 13 to 14). The level of hnRNP B1 mRNA produced is relative low and is only 2 to 5 % of the total hnRNP A2/B1 transcripts that are generated [178] (Figure 1.5).

To be fully functional, different posttranscriptional events have to occur to ensure the integrity of the protein. As in hnRNP A1 where CK2 seems to control the shuttling function of this protein by the phosphorylation of a single a.a [179], it has been suggested that the catalytic subunit of the protein kinase CK2 (CK2α) may interact with the hnRNP complex allowing phosphorylation of hnRNP A2 [180]. The other post-transcriptional event is the potential methylation of the arginine present in the RGG repeat of the GRD that may be accomplished by a mammaliam arginine methyltransferase, PRMT 1 [181]. This suggestion may be supported by the fact that it has been demonstrated that the utilization of methyltransferase inhibitor will lead to a predominant cytoplasmic proteins accumulation, which is also observed in certain tumors, suggesting that this posttranscriptional event may be related to oncogenesis [182, 183].

The level of expression of hnRNP A2 is not constant across different tissues. In fact, this protein is most abundant in the adrenal gland and the brain and a lower expression is observed in the heart, lung and intestine [175]. With its important role in RNA trafficking, hnRNP A2 is mainly involved as a shuttling protein between the nucleus and the cytoplasm. It is predominantly nuclear in localization, yet it can be be found in the cytoplasm, appearing as granules, like in oligodendrocytes [184]. In fact, actinomycin B treatment, which inhibits activity of the RNA polymerase II, allows accumulation of hnRNP A2 in the cytoplasm [185] , which demonstrate that it may be found also there.

Additionally, overexpression of cytoplasmic hnRNP A2 has been observed in certain tumors and has been suggested to be a potential tumor marker in different type of cancers, such as lung [186] and gastrointestinal [187]. Recently it has been proposed that that the A2/B1 expression ratio may be altered in tumor and may also be an indirect way for cancer diagnosis [188].

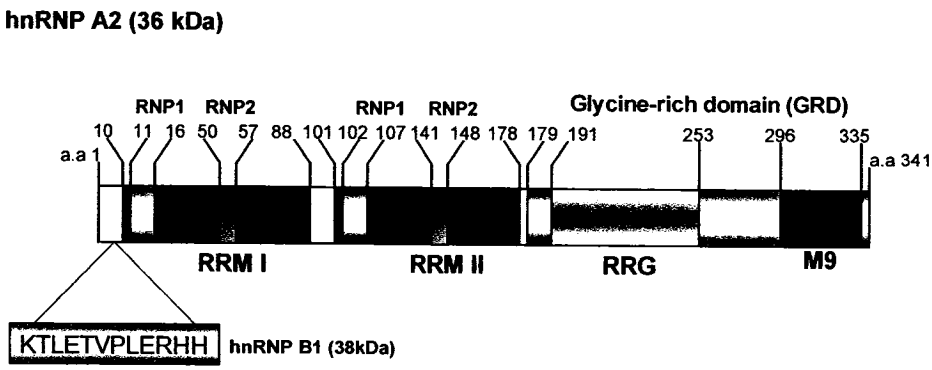


Figure 1.5 Functional Domains of hnRNP A2. The hnRNP B1 protein sequence is identical to the hnRNP A2 protein with the exception of a 12 amino acids insertion near the N-terminus.RNP1 and RNP2 are conserved motif present in the RNA binding motif RRM I and RRM II and their position are indicated here. The GRD domain follows the RRM and contains several repeats of RGG potentially involved in protein-protein interaction. The M9 are involved in nuclear export/import of this protein. Adapted from Mayeda et al. [169]

1.5.1.1 Functional domain of hnRNP A2

Members of the hnRNP group share several ressemblance and characteristic related to their potential to bind RNA. HnRNP A2 has two N-terminal RNA recognition motifs (RRM) (Figure 1.4) [169]. The fisrt element is named RRM I and is located from a.a. 10 to 88, and the second one, named RRM II, which may be involved in the interaction with the microtubules [189], is positioned from a.a. 101 to 178. It has been demonstrated that one of them is specific to the A2RE element and one that has non-specific activity, which binds other sequences and may be blocked by heparin *in vitro* [167].

Moreover, hnRNP A2 contains two regions highly conserved within the family and are named the ribonucleoproteins consensus sequences (RNP) and are positioned at a.a 11 to 16 and 102 to 107, for the first RNP element, and position a.a 50 to 57 and 141 to 148, for the second RNP. Those RNPs are located in the RRM motif of hnRNP proteins, who may contain more than one copy of RRM, like we observe in hnRNP A2 [169]. Following the RRM is a glycine-rich region (GRD), located in position a.a 179 to 341, and containing arginine-glycine-glycine repeat (RGG). The GRD has been suggested as a potential domain involved in protein-protein interaction with other RBPs or may also favor self-association [190]. Finally, one important domain which mediates the nuclear export/import is the non-canonical NLS M9 domain, located in position a.a 296 to 335. Present also in hnRNP A1 [191] and A3 [168], this domain may involve binding of hnRNP A2 to the import protein transportin 1 and binding of hnRNP A1 with RanGDP [168].

1.5.2 RNA trafficking system

RNA Trafficking is a complex process allowing transport of different mRNAs to sites where translation will occur. hnRNPs have been demonstrated to be involved in this trafficking processes and, more specifically, hnRNP A2 is involved in this process by interaction with cis-acting A2REs, which are present on certain mRNAs. The hnRNP A2 trafficking pathway can be summarized in a few steps: the nuclear transport, the assembly of granules, the transport of the granules on microtubules, and finally the regulation of the translation. To better understand the interactions between the different steps, a schematic overview is given in Figure 1.6.

Table 1.4 A2REs homology in mRNAs

Species	mRNA	Region	A2RE sequences
Transported RNAs			
Mouse	*mbp*	3'UTR	GCCAAGGAGCCAGAGAGCAUG
human	*mbp* RTS1	3'UTR	GCCAUGGAGGCACACAGC UG
Human	*mbp* RTS2	3'UTR	GCUGCAGAGACAGAGAGGACG
Rat	*MOBP81A*	3'UTR	ACCCCCGAGACACAGAGCAUG
Rat	*GFAP*	ORF	GCCAAGGAGCCCACCAAACUG
Mouse	*MAP2A*	ORF	GCCAAGGAGUCAGAAGACAUG
Rat	*ARC*	ORF	GCUGAGGAGGAGGAGAUCAUU
Human	*neurogranin* (RC3)	3'UTR	CCUGCGU CCCAGAGACUCCC
Human	*CaMKIIα*	3'UTR	UGCUGUGCCGCAGAGAUCCAC
Rat	*tau*	ORF	GCCAAGCAGGGAAA AGA UG
Other RNAs			
HIV-2	*tat, vpr*	ORF	UUGAAGGAGCCAGAGAGCUAC
HCV	*NS-5*	ORF	GCAAGGGGGCCAGAGAGCAUC
	Consensus		GCCAAGGAGCCAGAGAGCAUG
	A2RE11		GCCAAGGAGCC
	A2REmid		GGAGCCAGAGA
	A2RE3'		CAGAGAGCAUG
	Consensus	ORF	A K E P E S M

Adapted from Ainger et al. [163]

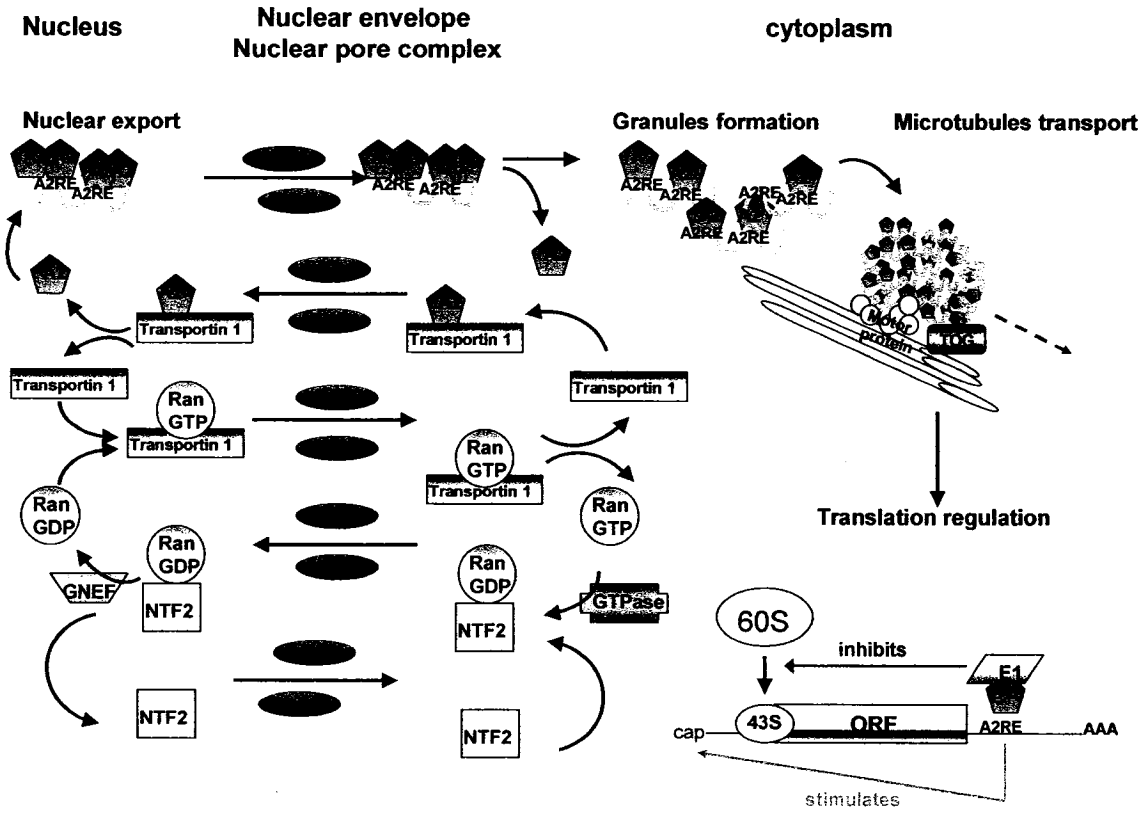


Figure 1.6 hnRNP A2 RNA Trafficking System. This system consists of the carrier Transportin 1 which allows translocation of the hnRNP A2 protein into the nucleus. Once inside, the hnRNP A2 protein may dimerizes and binds the A2RE transcript in order to fully saturate the element with hnRNP A2. The complex will then be exported out of the nucleus following the RanGTP system. In the cytoplasm, the low concentration of hnRNP A2 protein will favour monomer formation. However to ensure a full saturation of the A2RE site, an aggregation of the RNA will occur through the dimerization of hnRNP A2 to form RNP complex in combination with another cellular protein such as the kinesin, the dynein and the regulating protein TOG. This will allow the RNP complex to be transported along the microtubule. Binding of hnRNP A2 to A2RE stimulates the cap dependant recruitment of the 43s pre-initiation complex. To regulate the system, hnRNP E1 protein binds to hnRNP A2 and inhibits the recruitment of the 60s ribosomal subunit. Once at its destination, hnRNP E1 will dissociate and the 60s ribosomal subunit will be recruited and then the translation will start. More details are described in the text. Adapted from Carson et al. [176]

The first step of this pathway is the nuclear export. This step allows import and export of different macromolecules between the nucleus and cytoplasm. In the case of hnRNP A2, the protein will be brought into the nucleus, through a nuclear pore, by binding the transportin 1 molecule. Once in the nucleus the transportin-hnRNP A2 complex will then dissociate, which will allow recognition of the A2RE mRNA by the hnRPN A2 domain M9. The transportin 1 will bind Ran GTP to be exported out of the nucleus, to repeat the process [192]. The presence of Ran GTP in the nucleus is due an import process: Ran GTP, in the cytoplasm, will be converted to Ran GDP, by a GTPase, and then imported back into the nucleus by binding nuclear transporter signal 2 (NTF2). The Ran GDP-NTF2 complex will disassociate by addition of guanine to Ran GDP, by a guanine nucleotide exchange factor (GNEF). Dimerisation of the hnRNP A2 may occur, due to the high concentration of the protein in the nucleus, which will saturate the A2RE site and may help in the trafficking process [101].

Once the hnRNP A2 is in the cytoplasm, formation of the granules will take place. Since hnRNP A2 is at a low concentration in the cytoplasm, dimerised hnRNP A2 can dissociate itself and then be re-associated with A2RE-mRNA-bound hnRNP A2s [101]. Formation of these A2RE granules will require the assembly of about 30 A2RE RNAs [166] and involves a sorting system. In fact, the assembly of these granules is A2RE-sequence specific whereas the non-A2RE RNAs will be part of another granule population [176].

Following the formation of granules, a tug-of-war along the microtubules will take place. Each RNA granule contains motor molecules, such as dyenin and kinesin, which will move the granules toward the minus- or plus-end, respectively, of the microtubules. Going towards the minus end is called the retrograde route while going towards the plus end is called the anterograde route [101]. The composition of the RNA granules will affect the interactions with the different motor molecules, which will favor one route over another. In the case of A2RE-containing granules, they will generally follow an

anterograde route, whereas the non-A2RE containing granules will follow the retrograde route.It has been suggested that a tumor overexpressing gene, TOG2, from the big microtubule-associated protein family, interacts preferentially with the plus end of the microtubule [193], which is the anterograde route. Recently, hnRPN A2 and TOG have been identified as molecular partners [194] which may suggest a potential role for TOG2 in the transport system that delivers proteins to the cell periphery [195].

Different mechanisms for the suppression of translation of RNA transcripts, during the transport to their final destination, will ensure protien expression at the required sites of action. Such phenomena involve stabilization of the different transcripts, yet how this occurs is still not fully understood. It has been suggested that hnRNP A2 may be involved in translation by being a specific cap-dependant translation activator for A2RE RNAs, through recruitment of the 43s ribosomal subunit [196]. Moreover, the research teams of Dr. Carson and Dr. Barbarese observed that hnRNP A2 interacted with hnRNP E1, which had been suggested to inhibit the recruitment of the 60S ribosomal subunit. This association may explain the potential mechanism of translation regulation. It has been suggested that the A2RE granules, during their transport, will interact with hnRNP E1 to inhibit the translation process. This process is initiated by the recruitment of the 40S subunit due to the influence of hnRNP A2 cap-dependant translation. hnRNP E1 inhibits translation by stopping the recruitment of the 60s ribosomal subunit at the translation site. When the granules are localized at the proper cellular site, dissociation of hnRNP E1-A2 complex allows the activation of the translation by hnRNP A2. How the hnRNP E1-A2 complex dissociation is not understood, but may involve cooperation of different other factors [176].

1.5.3 hnRNP A2 and HIV-1

HnRNP A2 is an important cellular factor and it has been suggested to be involved at different levels in the HIV-1 replication cycle. However, the exact role has not been elaborated. To understand the role of this protein, we will study the interaction of hnRNP A2 with HIV-1. We will examine the role of this protein in the HIV-1 replication cycle.

1.6 Objectives of this project

In the literature it has been suggested that A2RE sequences have an important role in the process of RNA trafficking in the viral replication cycle [172]. We wanted to understand the role of hnRNP A2, which has been discribed to bind A2RE elements, in RNA trafficking. The objective of my M.Sc. project was to study the role of the hnRNP A2 protein in the replication cycle of HIV-1 and to examine the impact of its reduced expression on the viral cycle. To achieve this, we used the interference RNA technique to knockdown the hnRNP A2 protein using duplexes of siRNA [197-199]. We first optimized the siRNA technique, to ensure a good knockdown, before studying the impact of protien knockdown on the replication cycle.

In Chapter 3, the results of our different experiments will be presented. First, the development of the siRNA target and the response of the cell cycle to this treatment will be described. This entailed determining if siRNA knockdown interfered with the cell cycle and then the study of the effects of treatment on viral proteins, viral RNA transcripts, and virus production. Alterations of the genomic RNA localization lead us to study the potential association with different cellular components to try to elucidate the reason of this modification. Finally, the analysis and the discussion of this work will be presented in chapter 4.

Chapter 2 MATERIALS AND METHODS

In this chapter, I will describe the material and procedure that I followed to achieve objectives of this study.

2.1 Antibodies and Reagents

Mouse and Rabbit anti-hnRNP A2 were generous gifts of William Rigby (Darmouth Medical School, NH) and have been described in Bériault et al.[172]. A pan-hnRNP antiserum was generously provided by Benoit Chabot (Universtité de Sherbrooke, QC) as described in Bériault et al.[172]; rabbit anti-Lamp-1(#931B) were obtained from Minoru Fukuda (The Burnham Institute) and describe in Carlsson et al.[200]; rabbit anti-Rev R3 was generously given by Alan W. Cochrane; Rabbit anti-p24 was obtained from Trinity Biotech #cat 201 (Carlsbad, CA); rabbit anti-M6PR were generously offer by Paul Luzio (University of Cambridge, UK);rat anti-α-tubulin was obtained from Abcam (ab#6161); rabbit anti-myc was obtained from TechniScience (Montréal, QC) and used to detect an myc epitope-taggeg RILP expressor (pRILP-myc) a generous gift from Dr. Markus Thali (Universty of Vermont, USA) [201]. Monoclonal anti-GAPDH antibody was obtained from Research Diagnostic Inc (Flanders, NJ); anti-calnexin antibody (#SPA-860) was obtained from Stressgen (Victoria, CA); Goat anti-gp120 antibody was obtained from the NIH (National Institute of Health); Secondary fluorophore-conjugated antisera for anti-rat, rabbit and mouse (Alexa Fluor 488 and 594) was obtained from Molecular Probes; Secondary peroxidase-conjugated antibody was obtained from VWR (anti-rabbit # 111-035-003 and anti-mouse #515-035-003) and rabbit anti-goat antibody was acquired from Sigma.The hybridome for the 183 H12-5C antibody (against p24) was obtained from the NIH and the antibody was synthesized by Agro-brio (France); the anti-p24 biotinylated antibody (31-90-25) is a generous gift from Michel Tremblay (Université Laval, Qc) and the NIH reagent program. Nocodazole was purchased from Sigma-Aldrich. Moclonal anti-Stat-1 was a gift from Dr. John Hiscott (University McGill).

2.2 Cell culture

Hela cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Paisley, UK) supplemented with 10% of fetal bovine serum (Gibco Laboratories) and 1% of Pen/Strep antibiotics (Gibco Laboratories) at 37°C in a humidified atmosphere containing 5% CO_2.

2.3 siRNA

The duplex siRNA A2J (5'- AAGCTTTGAAACCACAGAAGA -3'; also known as A2.1), A2F (5'-AACCACAGAAGAAAGTTTGAG-3'; also known as A2.2) and siRNA A1.1 (5'-AATGGGGAACGCTCACGGACT-3') in a concentration of 25nM as described in Patry et al.[202] , was determined to produce the most significant knockdown of hnRNP A2 and hnRNP A1 gene expression. A non-silencing siRNA (catalogue # 1022076: 5'aattctccgaacgtgtcacga3') was purchased, as the siRNA A2J an siRNA A1.1, from Qiagen-Xeragon (Gaithersberg, MD) siRNA transfection were performed as described in Chatel-Chaix et al. [130] however HeLa cell were here used instead of 293T cells.

2.4 Transfection

Exponentially growing Hela cells were trypsinised and 3.0-3.5 x 10^5 cells were seeded into NUNC six-well plates with a cover slip present in the well for imaging analysis. 24 hours later, transfection of 25nM siRNA was performed on 70-80% confluent cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and Chatel-Chaix et al. [130]. Cells were transfected with either non-silencing, A2J or A1.1 siRNA. At 24 hours post-transfection, a second transfection was perfomed using 0.5μg of HxBru (*vif+, vpr+, vpu+, nef-*)[80] or 1μg of HxBru A2RE 2/1[172]. 30 hours later, the cells were washed with ice-cold PBS and lysed in NTEN buffer (100nm NaCl, 10nm Tris, pH

7.5, 1nm EDTA, 0.5% NP-40) for proteins analysis or fixed as described below for imaging analysis.

For experiments requiring a RILP expressor, 0.5µg of pRILP-myc was co-transfected with HxBru. Treatment with 5µM nocodazole before lysing the cell or the fixation was used to disrupt the mictrotubule network. Exposure of no longer than 30 minutes was performed to ensure that the integrity of the Golgi will be respected.

2.5 Western blot

HeLa cells were transfected and 30 hours post-2ndtransfection, were washed and lysed as described by Chatel-Chaix [130]. Cytosolic extracts were quantified for protein content by the micro-Bradford assay. Between 30-40µg of protein were equally loaded onto 10% denaturing polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane for 16 hours at 4°C and 100mA and then blocked with TBST (TBS and 0.05% tween 20) - 5% milk. Level of hnRNPs, pr55^{Gag} for HxBru, Rev and Env (gp120), were assessed by immunoblotting for 16 hours and glyceraldehyde-phosphate dehydrogenase (GAPDH) for 60 minutes at room temperature. Membrane was wash twice with TBST and incubated with the appropriate secondary antibody. Finally, the membrane was washed 4 times for 15 minutes and the presence of antibodies were reveled by enhanced chemiluminescence as describe by Yakunin et al. [203].

2.6 Immunofluorescence and Fluorecence in Situ Hybridazation (FISH) analysis

HeLa cells were transfected as described previously by Chatel-Chaix [130]. At 30 hours post-2nd -transfection, cell were washed with ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 20 min followed by permeabilization with 0.2% Triton X-

100 for 10 min. For cells that have been treated with nocodazole or with LMB prior the fixation, 2 to 5 more washes were performed to ensure that no residual chemical is present. To achieve immunofluorescence analysis, cells were washed with PBS pH 7.2 and blocked with 10% dry milk in PBS. Anti-p24 (to identify $pr55^{Gag}$ and its mature products) was used at a dilution of 1:400, anti-hnRNP A2 in a dilution of 1:200, anti-α-tubulin, anti-lamp-1 and anti-lamp-2 were used at a concentration of 1:250. Secondary fluorophore-conjugated antiserum (Alexa Fluor 488 and 594) was use at a dilution of 1:400.

For FISH/immunofluorescence co-analyses experiment, the FISH analysis was performed first. Following fixation and permeabilization, cells were treated with DNaseI (Invitrogen) for 30 min and washed once for 5minutes in PBS. The digoxigenin-labelled RNA probe was prepared as described previously by Bériault et al. [172]. In several experiments, an incubation of 15 minutes with the nucleic acid staining DAPI (1:500 in PBS) was done after FISH followed by 3 quick washes. Cells were mounted on microscope slides in 100 mg/ml Mowiol (Calbiochem), 25 mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma), 25% glycerol (v/v), 100 mM Tris-HCl, pH 8.5.

2.7 Imaging analysis

Imaging of cells was performed using an Olympus BX-51 fluorescence microscope from Carsen Group equipped with an UPlanFI 100x oil objective. Alexa fluor 488nm, 594nm and DAPI images were obtain by filtering the emission with 460-490nm, 510-550nm and 330-385nm bandpassfilters, respectively. Red, green and DAPI were photographed sequentially in black and white with Spot camera from Diagnostic Inst. using Spot advance software v.4.0.1 and Image-Pro Plus v.4.5.1. Images were then merged with Adobe Photoshop CS v.8.0 and then colored to their respective color. Phase contrast images using visible transmitted light were performed to verify the integrity and the morphology of the cells. Images were digitized and adjustments for all of the images were done equally for each of the channels to permit comparisons between the signals

intensities of the pictures obtained. All merged digitized images are imported into Adobe Illustrator CS version 11.0.0 for figures montage shown in this thesis. To ensure that the results that obtained are representative of our experiment, we captured at least 30 to 40 cells per experimental condition.

2.8 RNA extraction and RT-PCR analysis

HeLa cells were transfected as describe above. At 30 hours post-2nd-transfection cells were washed 3 times with DEPC-treated PBS and lysed on ice, for 30 minutes, using 100mM NaCl, 10mM Tris pH 7.5, 1mM EDTA, 0.5% NP-40 and a protease inhibitor cocktail (Roche Applied Bioscience). Lysates were spun for 30 minutes (13000 RPM, 4°C Biofuge Fresco Heraeux#3325B, Kendro Laboratory). An aliquot of 10% of the lysed supernatant was kept for western blots against hnRNP A1/A2, HIV-1 p24 and GAPDH. Before RNA extraction, cell lysates were treated with 40 units of RNaseout (Invitrogen) and 114 units of DNase I (Invitrogen) for 5 minutes at room temperature. Total RNA was extracted from cell lysates using Trizol LS (Invitrogen) according to the procedure suggested by the manufacturer. 5μg of glycogen (Fermentas) were used as carrier for RNA precipitation. Purified RNA was quantified by readingthe optical density at 260nm.

Resulting RNAs were subjected to RT-PCR amplification for GAPDH RNA, using a sense primer (5'-GCTGATGCCCCCATGTTCGT-3)' and an antisense primer (5'-CA.A.AGGTGGAGGATGGGTGT-3'), and for all three HIV-1 RNA species (2, 4and 9 kb) using primer combinations described previously [130], with the exception that a RNA PCR Core Kit (Applied Biosystems) was used. After 20 and 30 cycles, 10-15% of the total RT-PCR products was visualized on a 1% agarose gel using the Gel Doc system (Bio-Rad) and Quantity One software version 4.4.1 (Bio-Rad). For more sensitive detection of the HIV-1 spliced RNAs, 20% of RT-PCR product after 20 cycles was subjected to 2 additional PCR cycles in presence of 10μCi of [^{32}P] dCTP. Labeled PCR products were resolved on 5% denaturing acrylamide gel and detected by

autoradiography. Identity of the fragment obtained was compared with a radiolabeled 100bp DNA marker ladder prepared according the protocol suggested by Invitrogen as well as compared with the result of Purcell et al. [93].

2.9 Pelleting virus

At 30 hours post-2nd-transfection, the virus produced by the cells and present in the supernatant were firstly pre-cleared by a low speed centrifugation (Beckman centrifuge GR-6S) at 3000rmp for 20 minutes at 4°C. Then the cells were pelleted by ultracentrifugation (Beckman XL-80, rotor SW41) on 20% sucrose cushion in TN buffer (50nM Tris, 10mM NaCl, pH 7.5) for 1 hours at 35 000 rmp at 4°C. Pelleted virus was resuspended in TN buffer as describe by Russell et al. [204].

2.10 ELISA p24 assay

Virus quantification was done by an enzyme-linked immunosorbent assay (ELISA) in which the p24 capside protein was targeted, as describe by Bounou et al 2002 [205]. Briefly, a 96 wells plate (Immulon 2, Dynatech Ltd) was coated with a monoclonal anti-p24 antiboby (183 H12-5C) for 16 hours at room temperature. The plate was then washed 3 times with a solution of PBST (PBS and 0,05% tween 20)- 2% BSA (bovine serum albumin) Then different concentrations of a purified p24 recombinant (Research Diagnostics Inc., Flanders N.J.), in a linear range of 31,25 pg/ml to 2000 pg/ml, were used to establish a standard curve. Following 60 minutes incubation at 37°C with an anti-p24 biotinylated antibody (31-90-25), the wells were washed and reincubated with a conjugate of Streptavidin-peroxidase (Streptavidin-HRP-40, Research Diagnostics Inc.) for 30 minutes at room temperature. TMB-S (Research Diagnostics Inc.) was then added and the reaction stopped after 20 minutes by adding 1M H_2P0_4. Absorbance was measured at a wavelength of 450nm and virus concentration was calculated using the standard curve.

2.11 RNA extraction and Slot Blot analysis

HeLa cells were transfected as describe above. At 30 hours post-2ndtransfection virus was pelleted as described above. After a virus quantification (as describe above) an equal amount (2170ng) of the capside protein p24 was processed for viral RNA extraction as describe by Gough [206], but slightly modified. Briefly, cells were washed 3 times in DEPC-treated PBS and pelleted for 5 min at 1250 rpm and 4°C (Beckman centrifuge GR-6S). The supernatant was removed and the cell were resuspended in 200µl of cold 10mM Tris-HCL (pH 7.5), 0.15M NaCl, 1.5mM $MgCl_2$, 0.65% NP-40 and vigorously mixed by vortexing. After a 5 min centrifugation (13000 RPM, 4°C Biofuge Fresco Heraeux#3325B, Kendro Laboratory) the supernatant was carefully remove from the tube countaining pelleted nuclei and put in a fresh tube containing 200µl of 7M urea, 1% SDS, 0.35M NaCl, 10mM EDTA, 10mM Tris-HCL (pH 7.5). 400µl of phenol/chloroform/isoamylalcohol (50:49:1) was then added and vortexed. A 10 minutes room temperature centrifugation followed (13000 RPM, 4°C Biofuge Fresco Heraeux#3325B, Kendro Laboratory). In total two phenol/chloroform/isoamyl alcohol (50-49-1) extraction and one chloroform extraction were performed. The supernatant was then transferred in a new tube containing 3ul of glycogen (Boehringer Mannheim). Then 2 volumes of ethanol 95% was added and the mix was incubated for 16 hours at -80°C. Then a 15 minutes centrifugation (13000 RPM, 4°C Biofuge Fresco Heraeux#3325B, Kendro Laboratory) was done to pellet the RNA. The pellet was washed with 500µl of ethanol 70%. Followed with a 10 min centrifugation (13000 RPM, 4°C Biofuge Fresco Heraeux#3325B, Kendro Laboratory). With the supernatant discarded, the RNA pellets were resuspended in DEPC-treated water.

A slot blot analysis was perfomed to study the extracted RNA. An equal volume of GTC buffer was added to the virus and then mixed by inversion. After an incubation of 10 minutes on ice, centrifuge the mix for 5 minutes (13000 RPM, 4°C Biofuge Fresco Heraeux#3325B, Kendro Laboratory) and add DEPC treated SSC and formaldehyde to a final concentration of 6x SSC and 7,4% formaldehyde respectively and incubate for 15 minutes at 60°C. During this time, the nylon membrane was equilibrated in 20x DEPC

treated SSC. After the incubation, dilutions of the sample were prepared in 20x DEPC treated SSC and loaded on the membrane, which was sujected to a vacuum on the opposite side (to assist in absorption of the sample onto the membrane). After the membrane was washed three times with 20x DEPC treated SSC, once with 10x DEPC treated SSC-DEPC and let it dry completely. The membrane was autocross linked with a UV Stralinker 2400.

To hybridize our membrane, a fragment of 285nt was used from a PCR reaction to generate a ^{32}P-labeled cDNA probe able to detect all viral RNA species[207]; the 9kb, the 4kb and the 2kb. Two hours prehybridization, prior the hybridization, was performed in Church's buffer (0.5 M Na_2HPO_4 [pH 7.4], 7% SDS, 1 mM EDTA, 1% bovine serum albumin) at 65°C, to block unspecific binding of RNA. Then 16 hours hybridization, in the same buffer and where the probe was added, was performed at 65°C [80]. Following the hybridization, the membrane was washed four times in 2x SSC-0,1% SDS for 10 minutes at 30°C and then four times in 0,1xSSC-0,1% SDS for 10 minutes at 65°C. Finally the membrane was dried quickly and exposed to an autoradiographic film at -80°C. Alpha Imager apparatus and Alpha Ease SC fluorochem 8000 software version 3.1.2 was used to quantify the autoradiographic signals.

2.12 FACS

HeLa cells were transfected as describe above. At 30 hours post-2nd-transfection the cells were analysed for their nucleic content by fluorescence activated cell sorting (FACS) as describe [208]. Briefly, the cells were washed three times in ice cold PBS 1x and scrapped from their plates and then resuspended in ethanol 80% for 30 minutes at 4°C. Two more washes were done and then the cells were treated with 200 units of RNase A for 30 minutes at 37°C. Finally, the cell were treated with 30ug/ml propidium iodide in PBS 1X and then analysed by FACS. Propidium iodide is a stain that will incorporate dsDNA and has excitation wavelengths at 370 and 560nm and an emission wavelength at 631nm.

Chapter 3 RESULTS

3.1 Knockdown of hnRNP A2 protein expression by small interference RNA (siRNA)

To study the role of hnRNP A2 in the replication cycle of HIV-1, we used siRNA to decrease the expression of this gene and to look to its potential role in the trafficking of the viral RNA due to the presence of the A2RE element in the HIV-1 RNA.

3.1.1 Demonstration of the efficiency and the specificity of the siRNA target of hnRNP A2

Different sequences have already been described to decrease expression of this protein [202]. To ensure that we can get an efficient knockdown, different siRNAs were tested and results showed that the sequence A2-J was the most efficient and produces a significant knockdown of 80-85% on hnRNP A2 (Figure 3.1A). A negative control was used to ensure the specificity of the action of our sequence and, additionally, we also revealed the presence of GAPDH, an internal protein control, to demonstrate that the decrease observed here is specific to our hnRNP A2 protein knockdown.

Because most of the experiments that we had generated are based on a fluorescence microscopy technique, we also tested the sequence A2-J to validate this method in our experiment and to know if we can reproduce our result in immunofluorescence. As showed in figure 3.1B, where hnRNP A2 is represented in red and the nucleus in blue by DAPI, a decrease in expression is also present by the fact that we can observe a lower intensity of red in the cell that have been treated with the siRNA.

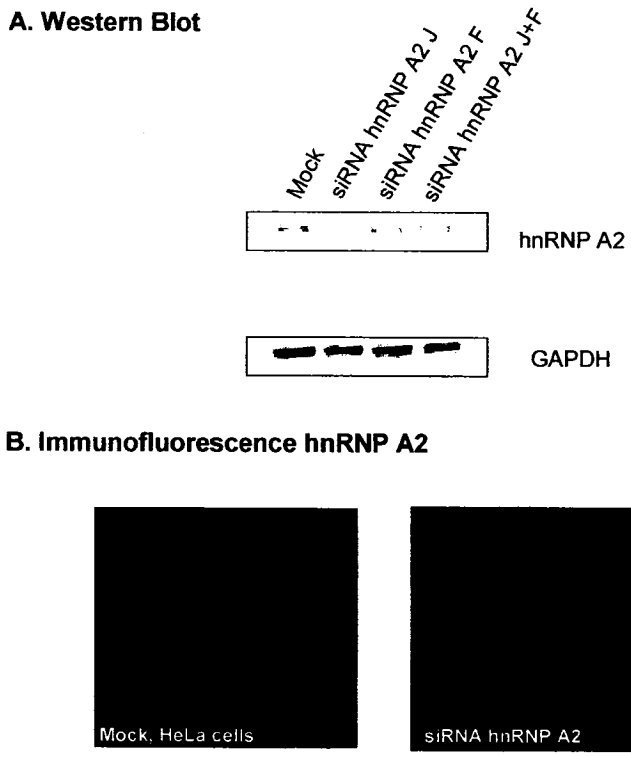


Figure 3.1 Efficient Knockdown of hnRNP A2 Protein. HeLa cells are non-transfected (mock) or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analyses are performed. **A.** Western blot analysis is performed by Dr. Poupon on total cell lysates. HnRNP A2 protein is revealed and compared with GAPDH level to observe the potential knockdown effect. SiRNA hnRNP A2 J sequence is the most efficient that the siRNA hnRNP A2 F ans the combinaison of the both with around 85% of knockdown. For the next siRNA experiment A2 J will be the one used. **B.** Immunofluorescence was performed on cover slip. HnRNP A2 protein, in red, is shown and compares with the nucleus staining, in blue. The cells treated with hnRNP A2 shown a 85% of succeddful knockdown.

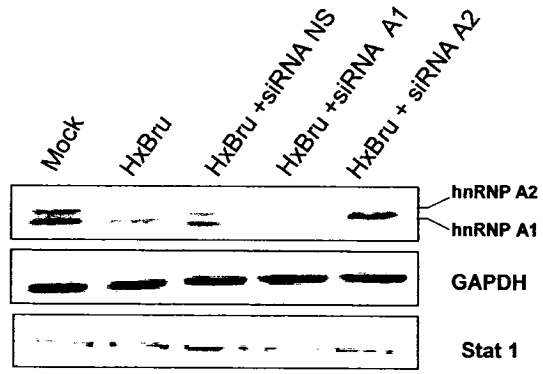
3.1.2 hnRNP A2 knockdown does not affect interferon signalling and the cell cycle progression

Activation of the interferon pathway following transfection of siRNA in cells has been described [209]. Moreover, recently it has been observed in HeLa cells that siRNA may alter the transcriptional profile of certain signal transduction genes related to proliferation and differentiation [210]. To ensure that our siRNA transfection does not induce such phenomena, we tested Stat1 expression. It is demonstrated that Stat1 is a protein involved in this process [211, 212] and has already been use as a marker for PKR activation in the interferon pathway [213]. PKR plays a role in IFN and dsRNA-signaling pathways by modulating the transcriptional function of STAT1 which will result in an overexpression of Stat1.

The cells were treated as described above and analysed 30 hours post-2ndtransfection. The expression level of Stat1 was determined by western blotting analysis. Since this protein is involved in the IFN patway, any change in his expression following our treatment will suggest a potential activation of this pathway. Results obtained here reveal no significant difference between the siRNA transfected cells and the control in terms of Stat 1 expression. This suggests that the siRNA treatment do not induce the IFN pathway. To guarantee that the transfection of siRNA and its efficiency was causing a decreased expression of hnRNP A2, we revealed the level of expression of hnRNP A2 by western blot and GAPDH was used as a control

To determine the potential side effects on the cell cycle, cell cycle analysis was previously performed by fluorescence activated cell sorting (FACS) by looking at the DNA content using propidium iodine. We then treated the cell as describe previously and, at 30 hours post-2ndtransfection, we analysed the sample. As we can observe on Figure 3.2B, siRNA treatments did not affect the different phases of the cell cycle and did not have an impact on apoptosis. To validate the test, simultaneous hnRNP A1 and A2 knockdown was performed and resulted in 50% of apoptosis as previously described [202].

A. Western Blot



B. FACS

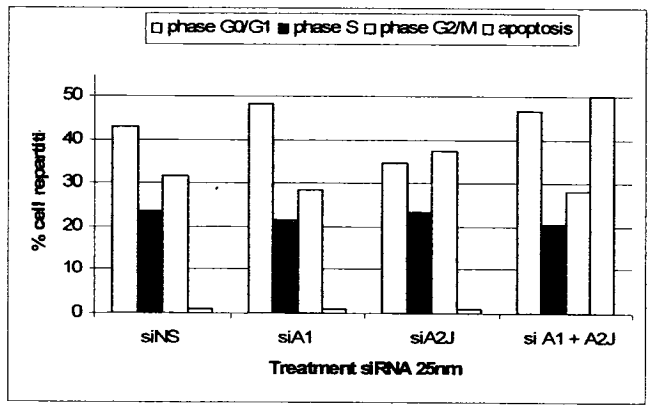


Figure 3.2 Cellular Responses to siRNA Treatment. HeLa cells are mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analyses are performed. **A.** Western blot analysis was performed on total cell lysates. HnRNP A2, Stat 1 protein is revealed to observe the knockdown and observe the potential INF activation respectively. Results obtained here do not show a cellular activation of the IFN pathway. GAPDH level is also revealed as a control for the amount loaded and is useful element to compare the knockdown. **B.** Cells are fixed in ethanol and treated with propidium iodine and analysed by FACS was performed by Dr. Poupon and are results of one experiment. No significant effect was observed excepted in the siRNA A1 and A2 treated cell.

3.2 Effect of the knockdown of the protein hnRNP A2 on the viral replication cycle of HIV-1

Following the optimisation of our siRNA system targeting hnRNP A2, we wanted to look at the potential effect of a decrease of expression of this protein on the replication cycle of HIV-1.

3.2.1 Expression of viral proteins in the cell

Since hnRNPs is involved in different cellular processes, such as mRNA localisation, translation and turnover [174], hnRNP A2 knockdown could then have a potential impact on the expression level of viral proteins. Protein expressions of the three different open reading frames (ORF) of the HIV-1 genome were then tested by western blotting (Figure 3.3A). One of them is the 9kb transcript, which expresses the Gag-Pol polyprotein (p160) and the Gag polyprotein ($pr55^{Gag}$), was analysed by looking at the expression level of $pr55^{Gag}$ and p24 (capside). No difference was observed following the siRNA A2 treatment. However, the siRNA hnRNP A1 treatment decreased for at least 50 % the level expression of $pr55^{Gag}$ and p24.

Next, the level of expression of gp120, an envelope protein express by the 4kb transcript, was examined. In addition, Rev, a protein express by the 2kb transcript, was analysed as well (Figure 3.3A). In either case, no significant impact is observed between each siRNA treatments and our control. This result suggests that the treatments done with siRNA, to decrease hnRNP A1 and A2, do not influence the viral protein expression produced by the 2kb and the 4kb transcripts. Finally, we looked at the level of the hnRNP A1 and A2 proteins to be sure that the results presented here are related to the siRNA treatment. Proteins were normalized using gapdh expression.

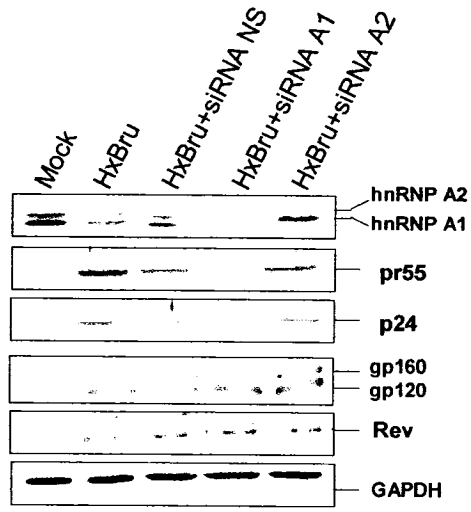


Figure 3.3 siRNA A2 treatment has no impact on viral expression protein. HeLa cells are mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analyses are performed. **A.** Western blot analysis is performed on total cell lysates. An efficient expression decrease is observed in siRNA A1 and siRNA A2 knockdown. No impact on viral expression is observed for the siRNA treatment at the exception of the siRNA A1 which decreased $pr55^{Gag}$ and p24 expression by at least 50%.

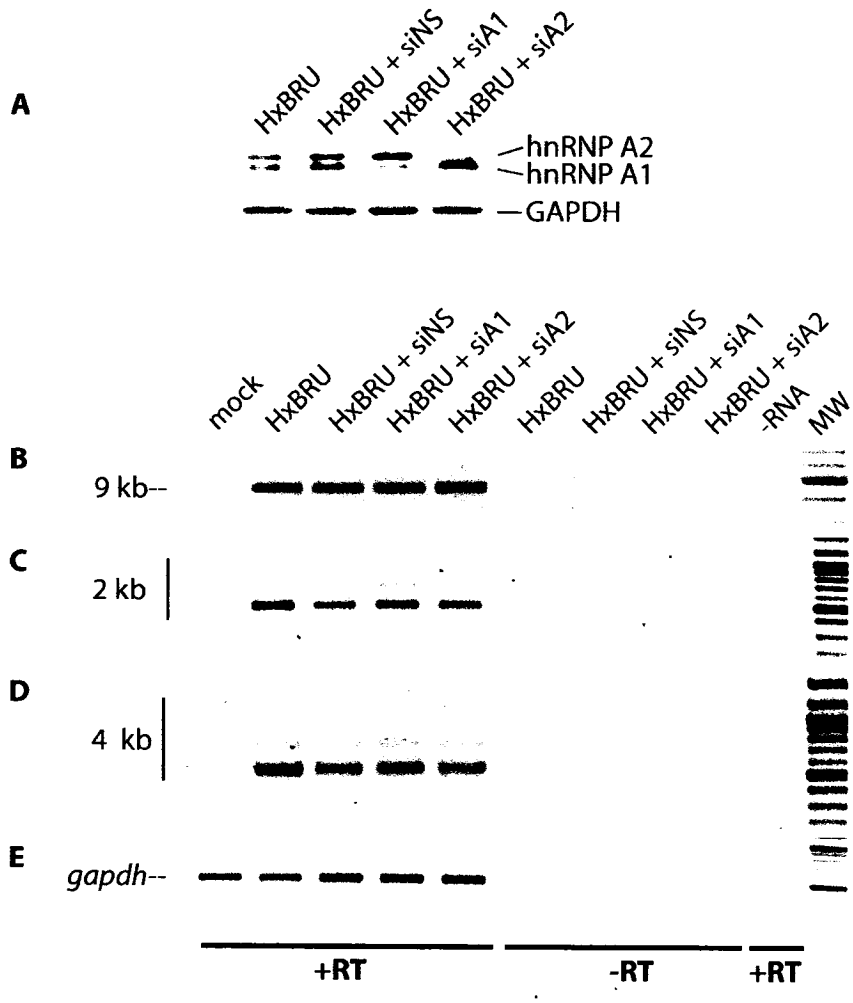


Figure 3.4 siRNA treatment has no impact on viral RNA transcript. A. HeLa cells were mock transfected (lane 1) or transfected with HxBRU proviral DNA alone (lane 2) or HxBRU and siRNA duplexes (siNS, non-silencing control, lane 3; siA1, siRNA to knockdown hnRNP A1 expression, lane 4; siA2, siRNA to knockdown hnRNP A2 expression (lane 5). GAPDH served as a loading control. **B.** HIV-1 RNAs were reverse-transcribed and PCR-amplified to identify i) 9kb (genomic) (ii), 2kb, and (iii) 4kb RNA species exactly as described (Chatel-Chaix et al. 2004). The expected size ranges of PCR products were obtained. gapdh RNA was used as a loading control for cellular RNA.

3.2.2 Level of the different viral RNA in the cell

In order to continue our analysis on the impact of hnRNP A2 knockdown on HIV-1, we performed RT-PCR splicing analysis on the different transcripts. Different hnRNPs have been described to be involved in vitro in RNA splicing. However our previous data does not suggest it [172]. Because we use another system then the one described by Beriault et al[172], we wanted to know if the splicing products of HIV-1 were affected by this knockdown in HeLa cells. Following the normal siRNA treatment and after verification of the efficacy of the knockdown of our experiment, which was as usual 80-85% or over (Figure 3.3), we performed the RT-PCR, as describe previoulsy, and ran the product on agarose gel and visualised the gel using ethidium bromide. The results demonstrated and confirmed that the decrease in the expression of hnRNP A2 does not affect the splicing of HIV-1 (Figure 3.4). The level of each of the 9kb, the 4kb and 2 kb transcripts were approximatively constant and were compared by our GAPDH control. During the last two cycles of the RT-PCR, radiolabelled dCTP were added and then, the products were visualized by autoradiography on a film. This result confirmed the pattern of the different splicing products observed previously following on polyacrylamide-urea gels (data not shown). However, even if hnRNP A1 has been already involved in different splicing event as with *SMN1* (spinal muscular atrophy) RNA [214] which leads to the development of the disease, the result demonstrated here does not show any effect of the siRNA A1 treatment on the splicing product of HIV-1, on either the 4kb or the 2kb transcript.

3.2.3 Localisation of the genomic RNA in the cell

Previously we demonstrated that mutations present in the hnRNP A2 response element were affecting the general trafficking of the 9kb transcript by blocking its export from the nucleus to the cytoplasm [172]. We have already shown that the general splicing pattern and the intracytoplasmic RNA trafficking was not affected; we then decided to look at the localisation of the genomic RNA in the cell. To achieve this, we combined an

immunofluorescence technique and fluorescence in situ hybridization (IF/FISH) assay; they will respectively reveal different proteins if using different antibodies and the HIV-1 genomic RNA if using a probe targeting the 9kb transcript. The normal procedure described above was performed and western blots were done to verify the efficiency of the knockdown and to view our internal GAPDH control (Figure 3.3A). However, following the 30 hours post-2ndtransfection, the coverslips present in the wells were fixed separately and treated for IF/FISH. Analyses demonstrated that in cells stained for hnRNP A2, in red, we observed a significant decrease of the protein expression, which was expected in the cells following siRNA treatment, compare to control (Figure 3.4). DAPI was also performed to facilitate the comparison between the samples and to localize the cells easily. Moreover, the FISH analysis which target genomic RNA, observed in green, revealed to us an important change in the genomic RNA localisation (Figure 3.5).In HxBru treated cells, we observed the normal distribution which are spread through the cytoplasm in a punctated pattern. However, presence of siRNA targeting hnRNP A2 was affecting the distribution of the genomic RNA which was observed in a specific perinuclear region. Compared with other controls, wild type and siRNA A1 knockdown, we found that this specific pattern was only observed in cells that were affected by the reduction of hnRNP A2 expression, in more that 90% of the case. In light of this specific accumulation, we used this phenotype to identify the subsequent siRNA hnRNP A2 knockdown that we will perform to maximise the different combination of immunofluorescence and FISH analyses.

3.2.4 Viral production

Following the previous observations, where an accumulation of genomic RNA in a perinuclear region in the cell was observed, we wanted to verify if the viral production was affected by the treatment. We analysed the supernatant by quantification of the virus particles released by an ELISA p24. A 25% decreased was observed between siRNA hnRNP A2 treated and the control as siRNA non-silencing and HxBru treated groups. The knockdown of hnRNP A1, previously described as a deficiency in Gag expression

(Figure3.6A), resulted in a 90% potential decrease of the viral production. This result was observed by the diminution of capside protein, the p24, released in the supernatent. With the presence of a low Gag expression in siRNA hnRNP A1 treated cell, we suspected that it may affect the viral production due to a potential lack of $pr55^{Gag}$ protein, which forms the future capsid and matrix of the virion and which will lead to a reduction in viral production. In contrast, hnRNP A2 siRNA treated cells where no difference in viral protein expression (Figure3.3A). However, there was a 25% decrease in viral production, suggestting a potential role of hnRNP A2 in assembly. Knockdown efficiency and gapdh, when tested, confirmed that there is an association between the results and the treatment performed (Figure3.3A). While measured by this assay, the viral protein produced may not show any difference in the viral particles production, nevertheless the RNA content may differ. The results obtained as to be confirmed by another technique because we may have production of VLP (virus like particles) that does not incorporate viral genomic RNA.

3.2.5 Viral RNA encapsidation

To know if the RNA content of the viruses produced is affected by the siRNA treatment; we decided to perform a slot blot analysis. As described earlier in the materials and methods, the cell were first transfected with siRNA, and a second transfection was performed with HxBru. This allowed time for viral expression. At 30 hours post-2^{nd} transfection, the cells were analysed for the efficiency of the knockdown. The viruses were then pelleted and the RNA was extracted, from a normalised amount of virus, to look at the potential difference. Results revealed no difference in RNA content for HxBru alone and HxBru siRNA non-silencing control. However, a potential increase of almost 40% in the RNA content was observed (Figure3.6B). Taking into account the previous results, there seems to be no difference in the production of viral particles yet the siRNA treated cells incorporate more RNA that the control by 1.4 to 1.6 times. Results presented here suggest that siRNA A2 treatment allow a increase in RNA content of the viral particles produced.

Immunofluorescence hnRNP A2 / FISH genomic RNA

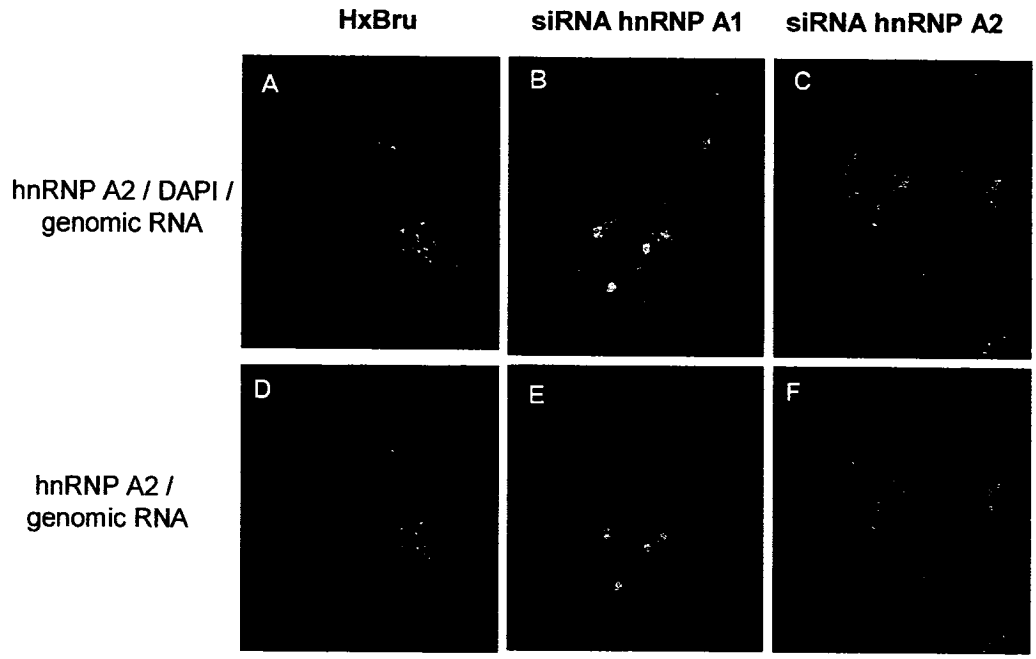
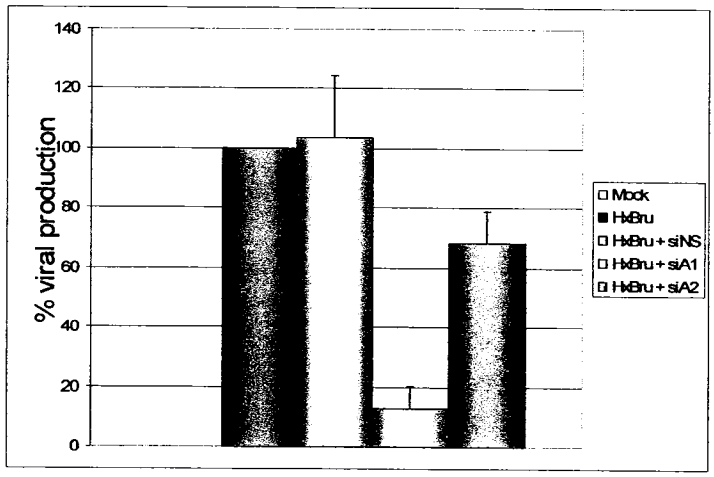


Figure 3.5 Treatment with siRNA A2 Results in a Perinuclear Accumulation of the Genomic RNA. HeLa cells are mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analysis performed. Cells are fixed and processed for immunofluorescence and FISH for hnRNP A2 protein expression, in red, and genomic RNA, in green, respectively. The upper row represents the hnRNP A2, DAPI, in blue, and genomic RNA staining and the lower one represent only the hnRNP A2 and the genomic RNA staining in order to emphasize on the hnRNP A2. Abundant expression of hnRNP A2 is observed in HxBru and HxBru siRNA hnRNP A1 treated cell is observed as the red pattern (A,B,D,E). hnRNP A2 knockdown was observed by the low expression of this protein (C,F). Genomic RNA is observed as a punctated pattern in HxBru expressing cell panel A and D and also in panel B and E represented by HxBru siRNA hnRNP A1 treated cell. In the cell HxBru siRNA hnRNP A2 treated, a specific perinuclear region accumulation is observed.

A. p24 ELISA



B. Slot Blot genomic RNA

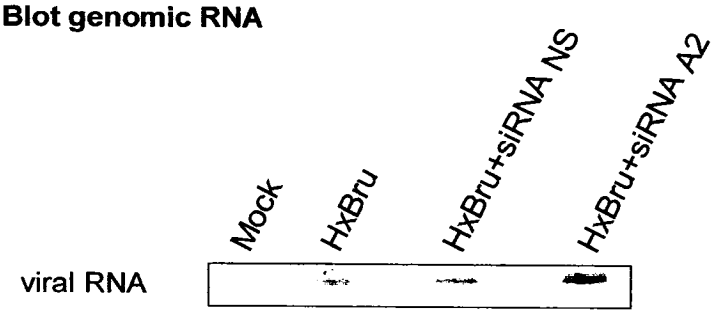


Figure 3.6 siRNA hnRNP A2 Treatment does not Affect Significantly the Viral Production but Causes an Increase of Viral Genomic Encapsidation. HeLa cells are mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analysis is performed. Supernatant are collected and cleared. **A.** p24 ELISA assay is performed. An important decrease is observed in siRNA hnRNP A1 protein, whereas a slight decrease of p24 was observed in hnRNP A2 protein. **B.** Ultracentrifugation is used to pellet the virus and then slot blot is performed on viral RNA content. A potential increase of 40 to 60% in the RNA content was observed in siRNA hnRNP A2 treatment.

3.3 The link between the perinuclar accumulation and cellular compartment

To better understand the accumulation in the perinuclear region, we attempted to identify different proteins and cellular compartments, using an immunofluorescence and fluorescence *in situ* hybridization techniques.

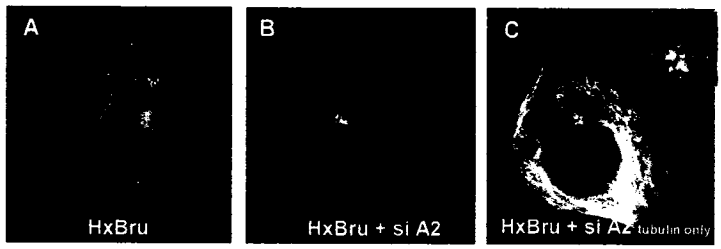
3.3.1 Identification of the site of accumulation in the perinuclear region

To understand why the accumulation of genomic RNA is occuring in the perinuclear region, we need to determine a link(s) between the components related to this region. A close observation of the nucleus and of the site of accumulation suggests that it might be associated with an important intracellular structure. The bean-like appearance of the nucleus is due to the pressures exerted on it from the different cellular organelles as well as the presence of the MTOC, which is the anchoring point of the microtubule network. Many cellular organelles tend to cluster around the microtubule organizing center (MTOC), typically found in a perinuclear position, due to microtubule-mediated transport. However, certain organelles can be found in a more dispersed distribution, suggesting that they are not necessarily being actively recruited toward the MTOC. We wanted to look at the potential interaction of the genomic RNA with the MTOC, since it has already been suggested that viral assembly involves the microtubule network [165]. To accomplish this, we performed IF/FISH on the microtubules using an anti-α-tubulin antibody, revealed in red, which will be compared with the genomic RNA, revealed in green. We performed DAPI staining to facilitate cell localization and to delimit the nucleus, which will help us locate the perinuclear region. The results demonstrated that the region of genomic RNA accumulation coincided with the MTOC (Figure 3.7A).

To determine the effects of this accumulation on different cellular compartment, related to the MTOC, we performed IF and FISH, in tandem. The different cellular compartments will be revealed in red, while the genomic RNA will be revealed in green. (Figure 3.7B). DAPI was also performed to identify the nucleus. Furthermore, different

compartments are known to be related to this particular perinuclear region such as the Trans-golgi network, the endoplasmic reticulum, and the endocytic recycling compartment. To look if the accumulation was located at the endoplasmic reticulum (ER), we stained for calnexin, an endoplasmic chaperone which controls the release of partially folded glycoproteins in the endoplasmic reticulum [215]. Because this cellular compartment is spread throughout the cell, we were not able to associate clearly the endoplasmic reticulum as the site of our genomic RNA accumulation. We continued our analysis with another cellular compartment, the Golgi. We performed IF against the manose-6-phosphate receptor (observe in red), known to be a marker of the trans-Golgi network (TGN) [216]. Only a partial and irrelevant co-localization was observed. However, this lead us to look at the late endosome, which may be linked to the trans-Golgi network [217] and is also known to be involved in viral assembly in macrophages (as previously stated). Finally the Lamp-1 compartment, which has been described participating at the site of viral assembly in macrophages [218], was studied by IF using an anti-lamp-1 antibody, revealed in red. We observed a potential association, at the site of accumulation, between the genomic RNA and vesicules stained for Lamp-1. Each immunostaining was compared with the HxBru siRNA non-silencing control.

A. Immunofluorescence tubulin / FISH genomic RNA



B. Immunofluorescence / Fish genomic RNA

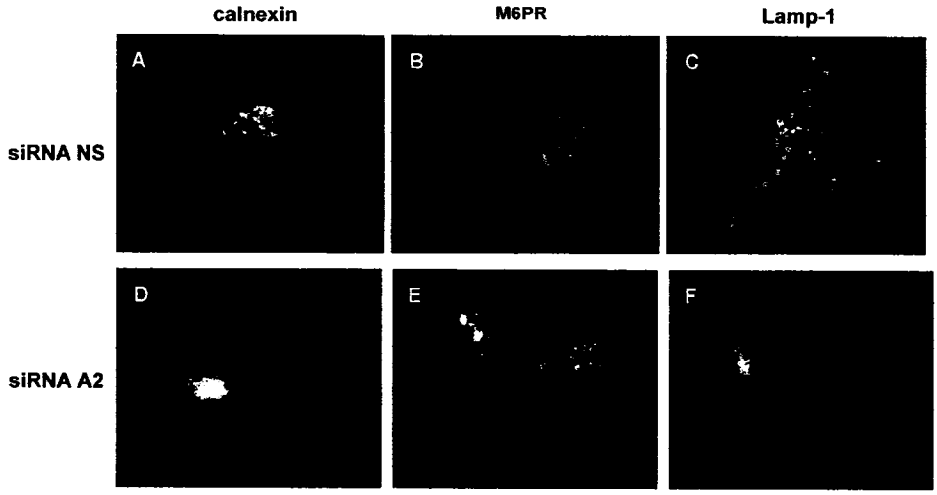


Figure 3.7 Genomic RNA Accumulate at the MTOC and are not Closely Associated with Cellular Membrane Compartment. HeLa cells are mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analyses were performed. Cells are fixed and processed for immunofluorescence for protein expression, shown in red, coupled to FISH for genomic RNA, in green, respectively. **A.** Tubulin is shown in red. An accumulation at the MTOC of genomic RNA is seen in siRNA hnRNA A2 treated cells compared with the HxBru virus alone. Pannel B and C were analysed by Dr. Poupon **B.** Different cellular compartments are stained in red and compare to associate the accumulation to a potential compartment. Only partial overlap is observe in the case of the trans-Golgi with the Manose-6-phosphate receptor (M6PR) and with the late endosome represented by lamp-1. Pannel A, B, D and E were analysed by Dr. Poupon.

3.3.2 The perinuclear region seem to be associated to the MTOC

To demonstrate the association between the RNA accumulation and the MTOC at the perinuclear region, we performed nocodazole treatment, known to disrupt the microtubule network and release the component associated with it. Following the 30 hours post-2nd transfection, we then treated the cells with nocodazole for 30 minutes, and then fixed them and performed the regular IF tubulin (in red)/FISH (in green) analysis (Figure 3.8). We then observed that the genomic RNA accumulated at the MTOC was slightly more diffused then the normal control without nocodazole. To make certain that the siRNA was efficient, that HxBru was well expressed and that we loaded an equal amount of protein, WB analysis was perform and the 85% knockdown was observed (data not shown). The results suggest that the accumulation observed was related to the MTOC and suggest a potential relation between the genomic RNA and microtubules.

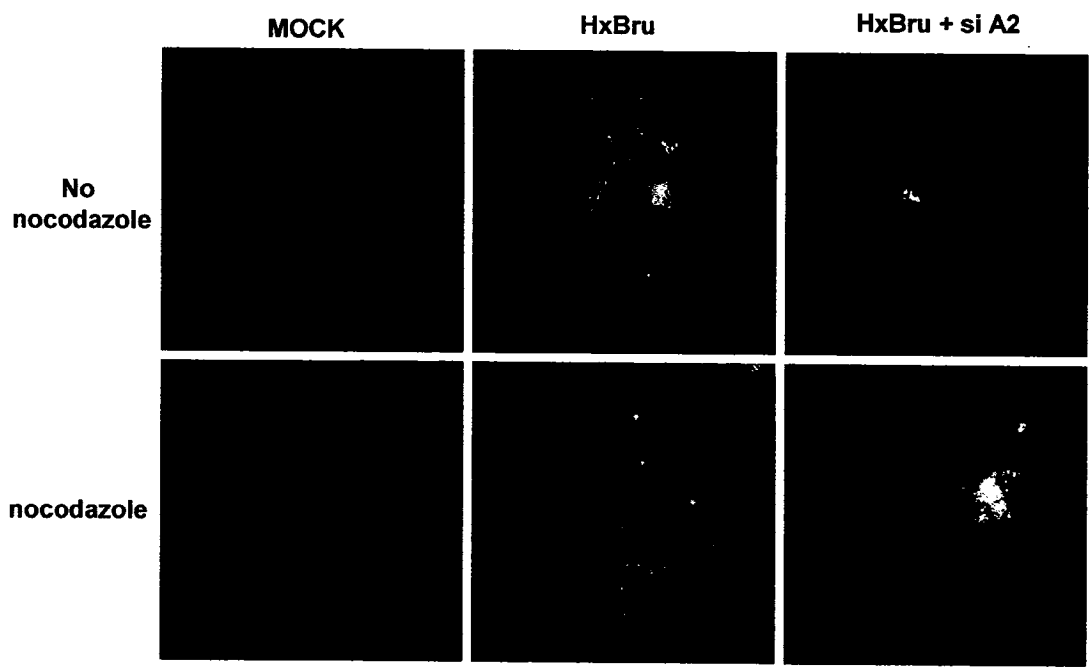


Figure 3.8 Microtubule Disruption Affects the Accumulation Through a Small Diffusion. HeLa cells are mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analyses are performed. Cells are treated or not with nocodazole for 30 min and then fixed and processed for immunofluorescence and FISH for tubulin, in red, and genomic RNA, in green, respectively. Samples treated with nocodazole (bottom row) demonstrate microtubule disruption where the punctuated pattern is observed compare to the non treated cell (upper row) where microtubules are represented like as a spider web-shaped.

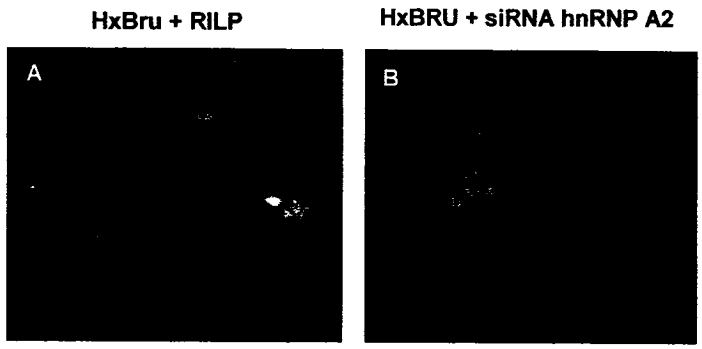
3.3.3 Potential interaction of hnRNP A2 with cargo molecule associated with microtubules network

Until now, the data suggest that the accumulation was related to the MTOC. Due to the important role of the microtubules in the cell, we tried to elucidate how and which mechanism was involved in this perinuclear accumulation at the MTOC. As mentioned earlier, different proteins are know to act as cargo for transport. To understand which mechanisms could be involved and to reconfirm the hypothesis that the genomic RNA is dependant on the microtubule system, we overexpressed Rab-interacting lysosomal protein (RILP), a Rab7 effector protein. This protein is known to recruit the dynein-dynactin motor complex to the late endosomes/multivesicular bodies at the MTOC [201]. Overexpression of RILP was achieved by transfection of a RILP expressor in presence of HxBRu. Then the regular IF/FISH analysis and controls analyses were performed in parallel with HxBru siRNA hnRNP A2 knockdown.

This experiment demonstrated a clear redistribution of the genomic RNA with the RILP expressing vector. In fact, RILP overexpression coordinates the transport of Rab 7 and the recruitment of the dynein motor complex, which results in the recruitment of the genomic RNA at the perinuclear region. In both case, in the cell expressing RILP+HxBru and in the HxBru+siRNA hnRNP A2 treated cell, we observed a similar effect of genomic RNA accumulation (in green) at a perinuclear region (Figure 3.9A). A co-IF of anti-myc was performed to determine RILP localization and to ensure that RILP overexpression was responsible for the phenotype observed in RILP expressing cells. In tandem, we performed an anti-Gag stainning, in red, to look at the Gag expression and ensure HxBru transfection (Figure 3.9B). These results suggest that RILP overexpression will favour recruitment of Rab 7 and a potential localisation of genomic RNA at this same site. This could also indicate that hnRNP A2 may act on the microtubules network. To verify that the results are relevant, we first looked if the HxBRu vector was expressed by looking at $pr55^{Gag}$ and p24 proteins expression. Next, we looked at the efficiency of the hnRNP A2 knockdown compared with the level of GAPDH protein loaded (Figure

3.9C). Finally we comfirmed the presence of Rilp-myc tagged by looking at the expression of myc (data not shown).

A. Immunofluorescence hnRNP A2 / FISH genomic RNA



B. Immunofluorescence

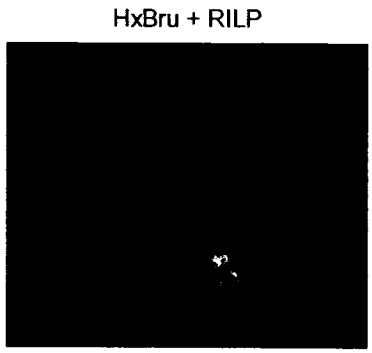


Figure 3.9 siRNA hnRNP A2 Treatement and RILP Overexpression Result in a Perinuclear Accumulation. HeLa cells sre mock transfected or transfected with HxBru and 25 nm of siRNA. 30 hours post-transfection analyses are performed. Cells are fixed. **A.** Immunofluorescence targeting hnRNP A2 is in red and FISH for genomic RNA in green. In both, we detected the genomic RNA distribution in HxBru and RILP transfected cell compare to HxBru siRNA hnRNP A2 treated cell. A similar perinuclear accumulation is observed in both cases. **B.** Co-immunofluorescence is performed so as to confirm HxBru expression by targeting p24 in red and to look at the localization of the RILP-myc expression in green.

Chapter 4 SUMMARY AND DISCUSSION

4.1 HIV-1 genomic RNA trafficking and viral assembly

With siRNA targeting hnRNP A2, we investigated the effect of protein depletion in HIV-1 expressing cells. We first tested different siRNA sequences to get the optimal decrease in expression, which resulted in an 80-85% knockdown (Figure 3.1), and we ensured that we did not induce the interferon pathway or affected the cell replication cycle. No significant effect was observed on the cell cycle (Figure 3.2); we then continued our investigation and looked effect of the knockdown in HIV-1 expressing cells.

Different hnRNPs are involved in the translational process. Recently, hnRNP A1 has been demonstrated to be a novel trans-acting factor that modulates alternative initiation of translation of the fibroblast growth factor 2 mRNA [219]. Moreover, the *Drosophila* homologue Hrp48 is known to bind and regulate translation of oskar mRNA [220]. To exclude the possibility that hnRNP A2 is not involved in the translation mechanism, we examined the level of expression of the viral proteins. Due to the presence of three different open reading frames, we looked at generating the 9kb, 4kb and the 2kb transcripts. We then looked at the protein expression of each of those transcripts. Results obtained demonstrated that the siRNA targeting hnRNP A2 showed no significant difference in the expression pattern of the viral proteins (Figure 3.3A). On another hand, a drastic expression decrease was observed in siRNA hnRNP A1 treated cells. This significant effect of siRNA knockdown may not be related to any splicing defects, suggested by the results obtained by RT-PCR. The low expression of $pr55^{Gag}$ and p24 proteins may then be related to their stability or even to their regulation. Involved in different cellular events, hnRNP A1 was recently described to interact and influence the internal ribosome entry site (IRES). By targeting hnRNP A1 using siRNA, it was specifically inhibiting translation at different IRES-dependent initiation codons [219]. Moreover, different studies have demonstrated the potential role of hnRNP A1 in retroviral regulation of post-transcription, such as in down-regulation of transcriptional

activity of Rex protein of HTLV-1 [221]. However in our case it seems to be mostly a cooperation with the cis-acting regulatory elements (INS) that impairs stability, nucleocytoplasmic transport, and translation [222, 223] . Furthermore, the presence of hnRNP, bound to the RNA, may protect the RNA against degradation from RNAses, by masking cutting sites.

We then determined if the siRNA targeting hnRNP A2 was affecting the different transcripts, we performed RT-PCR (Figure 3.4). No major differences in the levels of RNA expression, which is consistent with previously published data [172]. This confirms that hnRNP A2 does not act on splicing of HIV-1 and suggest again the idea that it may be involved in trafficking. As mentioned earlier, hnRNP A2 is the key element in the trafficking mechanism of different mRNAs. From the results obtained, it seems hnRNP A2 was also involved in HIV-1 trafficking. In fact, incorporation of a single point mutation in the position A8G of the A2RE of the genomic HIV-1 RNA disrupts the interaction of the protein hnRNP A2 and its element, which confined the RNA in the nucleus. We wanted to determine if this localisation of the genomic RNA will be affected by siRNA A2 treatment. To achieve this, we combined an immunofluorescence technique, which reveals different proteins bound to different antibodies, and a fluorescence in situ hybridization (IF/FISH) assay, which reveals the HIV-1 genomic RNA by a probe targeting the 9kb transcript. Observations lead us to identify a specific perinuclear accumulation site which seems to be specific to the hnRNP A2 knockdown treated cells. In fact no significant accumulation was observed elsewhere in our controls, where a cytoplasmic punctuated pattern was observed (Figure 3.5). Even if hnRNP A1 was previously mentioned to have an impact on $pr55^{Gag}$ expression, it does not seem to affect genomic RNA localisation.

Observation of this genomic RNA distribution could have an impact on the viral replication. We then looked at the viral production. In fact, the presence of the genomic RNA at this specific region should affect the viral production, since the genomic RNA may potentially not be encapsidated and sequestered at this point. Following a p24 ELISA (Figure 3.6A), we observed a significant decrease in viral production in siRNA

A1 treated cells, which we suspected to be due to the low production of $pr55^{Gag}$ protein, which is essential for virus formation. If CA, MA and other proteins cannot be produced, there will be no virus since there are no viral structures produced. However, a 25% decrease was observed in siRNA A2 treated cells. At first sight, we may think that hnRNP A2 knockdown does not affect viral production, but since this technique is based on p24 protein detection, it does not differentiate between RNA-containing virus and virus which does not contain RNA. To differentiate normal virus from the VLPs, we performed a slot blot to analyse the genomic RNA content (Figure 3.6B). In hnRNP A2 depleted cells, a 40% increase of the genomic RNA incorporation was observed compared with HxBru. This observation is consistent with the potential role of hnRNP A2 in viral trafficking. In fact different models were suggested in which this protein may be involved in trafficking in different organism [194]. However, the increased RNA concentration was surprising since our first hypothesis was that the depletion of hnRNP should potentially decrease the viral RNA content in the virus, due to its sequestration at the perinuclear region.

To understand the cause and the relation of the perinuclear accumulation of the genomic RNA, we looked more closely at this region. A close study of the nucleus at the site of the accumulation suggests that it may be associated with an important structure. In fact, when we looked closely at the nucleus, we observed a bean shape, due to the presence of different pressures exerted on the nucleus by the presence of different organelles and the MTOC. As mentioned earlier, MTOC is an important point of convergence where different RNP granules will follow its route to their destination. Since the observation of the perinuclear accumulation was potentially occurring at the same site, we wanted to know if is the MTOC and its related cellular compartment may be related or involved in this accumulation. Fluorescence in situ hybridization and immunofluorescence co-analysis were performed to attempt to associate potential cellular compartments (Figure 3.7) with the genomic RNA. Following the siRNA against either for hnRNP A1 or hnRNP A2 treatment and HxBru transfection, we wanted to study if this accumulation was related with the microtubules. The immunofluorescence performed revealed that this accumulation co-localized with this network, which suggested a relationship with the

MTOC. To confirm this, we demonstrated that when the cells were treated with nocodazole, a slight diffusion was observed, which suggested again the potential relationship of the perinuclear accumulation with the microtubules (Figure 3.8). The microtubule network is involved in different mechanisms and it has been suggested that HIV-1 uses this route, and the MTOC, during transport from the cytoplasm to the nucleus and vice versa [224] . Coincidently, during the writing of this thesis, it was shown that ψ+ RNA, acting as a subcellular localisation signal to the centriole, may be a structural facilitator of Gag assembly [225]. These observations support the conclusions that the perinuclear and the MTOC regions are important in viral assembly, hence supporting the rationale behind our work. Futhermore, with this recent study, we can suggest an explanation to why there is no virus production in cells treated with hnRNP A1 siRNA: since Gag is not expressed in hnRNP A1 cells, the ψ+ RNA will not be sequestered by Gag and no viral assembly will then be possible.

RNP complexes, moving along the microtubules, are in a state of continual flux since their protein composition will vary they complete their journey to the cell membrane. Moreover, formation of these complexes allows mRNAs transport to their destination. This involves different motor proteins proteins, such as dynein and kinesin, which are locked in a continuous struggle due to their opposing activities. Furthermore, protein interactions with the motor proteins, in the RNP complex, may be critical in stabilizing the system and favouring one route over another. To know which mechanism may be involved with the perinuclear RNA accumulation and to confirm the idea that HIV-1 is associated with microtubles, we compared our system with another system that resulted in a similar accumulation. In fact, over expression of the Rab7-interacting lysosomal protein (RILP), we get a perinuclear accumulation, which may suggest that a similar mechanism is at play. Actually, when overexpressed, RILP is known to coordinate the transport of cargo protein and to recruit dynein motor complexes to the endosome/multivesicular bodies at the MTOC. In this sense, over expression of this protein will allow stabilization of molecules involved in the retrograde pathway and will consequently favour the complex to be rerouted to the minus end of the microtubule, which corresponds to the MTOC. Furthermore, we can hypothesis that hnRNP A2 may

also be related to a similar pathway, but in a different manner. In fact, presence of overexpressed RILP stabilised the dynein complex, resulting in an accumulation at the MTOC. On the other hand, the perinuclear RNA accumulation observed (Figure 3.9) is the result of hnRNP A2 protein depletion. As a result, we can hypothesize that hnRNP A2 may be related in the stabilisation of the anterograde pathway. Consequently, the depletion of this protein may destabilize the trafficking process and then rerouting the genomic RNA toward the MTOC.

Up to now, we suggest a close association with the microtubules and the RNP-containing genomic RNA and we also suggest that hnRNP A2 may play a role in the anterograde pathway, by interaction with TOG2. Recently, it has been demonstrated that TOG2 and hnRNP A2 would interact together, potentially being molecular partners [194]. Since TOG2 interacts preferentially with the microtubule plus end, TOG2 would then related to the anterograde route toward the cell membrane and may also be involved in protein delivery to the cell periphery [193, 195]. This potential interaction between TOG2 and hnRNP A2 is supportive of the data obtained during our studies. In fact, the presence of hnRNP A2 protein, which bound the A2REs present in the HIV-1 genomic RNA [166], will allow formation of a RNP complex with TOG2 which will, in cooperation with kinesin, act on the anterograde pathway and the transport of the RNA to the cell membrane. However, depletion of the hnRNP A2 protein would perturb the whole process, because the genomic RNP complex would not be associated with the TOG2 protein. This lack of association would render the anterograde pathway, where the kinesin acts, weaker towards the opposite force of the dynein. This would result in rerouting of the RNA on the retrograde pathway and perinuclear accumulation at the MTOC.

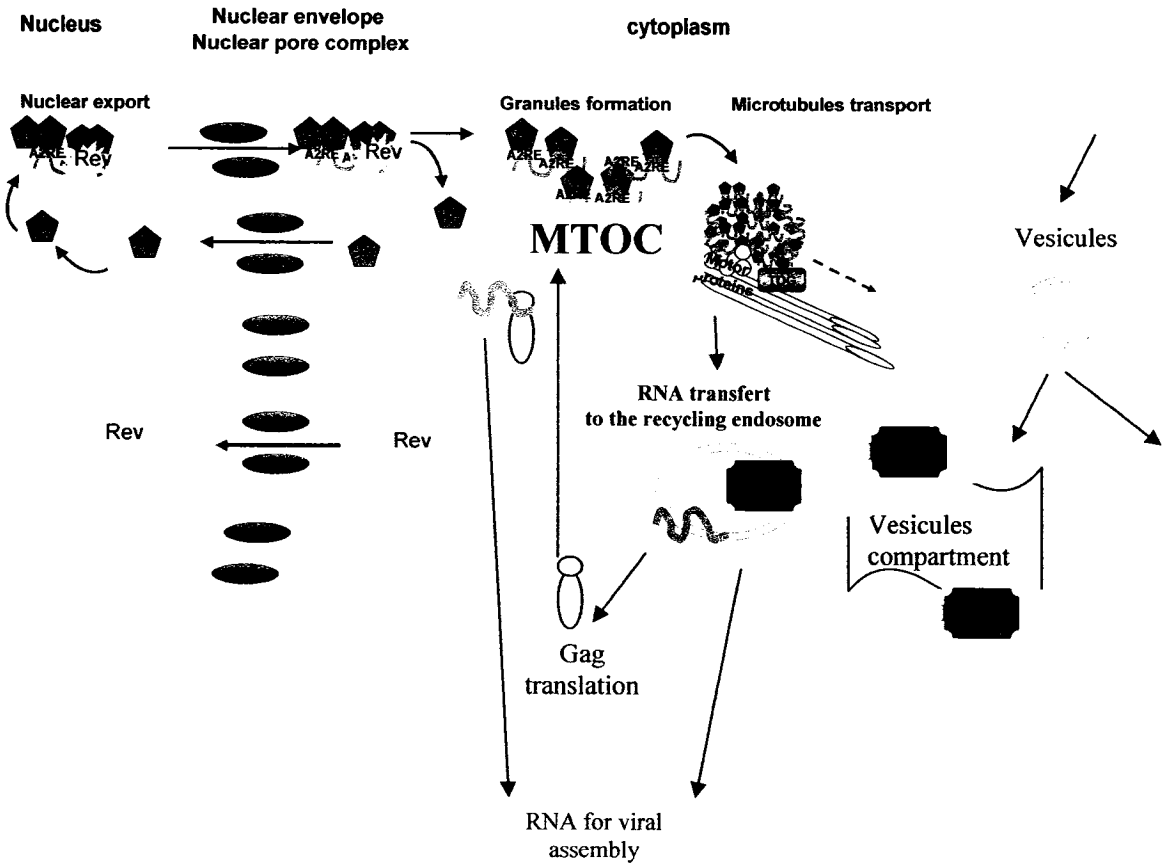
As mentioned earlier, the RNP complex and membrane may use the network as a center for vesicular trafficking. Previous studies suggested HIV-1 may follow the same route and an example of this is the murine leukemia virus [165]. The further proposal suggests that the trafficking of genomic RNA may be using RNP complex on recycling endosomal vesicles, in a microtubule dependent manner. This hypothesis is interesting because it

could explain our results where we get an increased presence of genomic RNA in virus. We are suggesting that the viral assembly may start on vesicles along the microtubules. In this sense, MTOC may be a potential starting and meeting point for the vesicles and the viral components for viral assembly. The presence of a high genomic RNA concentration at the potential starting point may be an issue because more RNA transcripts could be taken up for encapsidation [225]. We try to integrate these concepts in our system, to see if RNA trafficking can be achieved on vesicles which may potentially interact with the microtubules. We investigated further different vesicular cellular compartments involving this interaction. We then tested ER, however no colocalization was observed with calnexin, an endoplasmic chaperon which will interact with partially folded glycoproteins in the ER to ensure that the proteins are correctly folded. In fact, the wide repartition of ER in the cytoplasm was not closely associated with our accumulation. We tried to identify the compartments that were more restricted to this region since our accumulation is specific to a region. The cellular markers of the trans-Golgi network and the late endosome were respectively tested. As a result, a small overlap and partial co-localisation were observed. Taken together, those results suggest that the late endosomal pathway, the MVB and endosomal compartments, may participate in someway with RNA localisation and trafficking in viral assembly. It might be suggested that the potential viral particle formation may occur in a similar way as in the macrophages using MVB and the vesicular sorting pathway for viral budding [48, 136], but this is controversial. This hypothesis may also suggest why we may observe a possible 40% increase of the RNA in the viral particles while the MVB is passing through the accumulation site. By doing so, if the viral particle is formed there, more RNA at the formation site will be reflected in the particles because more RNA will interact with Gag and then be encapsidated. However, the partial localization and the overlap obtained with Lamp-1, which is a marker of the late endosome MVB, does not suggest that this system is involved in viral particle released. But to eliminate this suggestion, electron microscopy can be performed to verify the presence, or not, of viral particles in the periphery of the MTOC. Moreover, since no other cell types were demonstrated to use the MVB to release the viral particles, this hypothesis is not favored. However, if we look at previous work done on MLV and at our data, we could suggest

that HIV-1 may interact with cellular membranes, such as vesicles, to be routed at the cell plasma membrane in a certain way. In fact, the data suggests that MLV and genomic RNA are recruited at the endosome, trafficked on the Rab vesicle, which is involved in the endocytic recycling pathway. This trafficking is done in a Gag-Env dependant manner and then the complex is routed to the plasma membrane for viral assembly [165]. Since both Gag and Env have been describe to possess an endosomal targeting signal [226], viral assembly may then occur on the endosome. The data obtained here does not suggest a strong association with the late endosome, but it does not rule out the potential interaction with the recycling compartment. Taken together, those results suggest a potential mechanism for viral RNA trafficking. However, this model does not suggest the presence of an increased genomic RNA content in the virus. But recently, it has been demonstrated that Gag and RNA interact with each other at the perinuclear and centrosomal sites, which are the sites of our genomic accumulation under our hnRNP A2 knockdown [225]. In that case, the RNA content could be directly influenced potentially by the increasing interaction of Gag and the RNA at the MTOC. A distinct model can not be proposed, since more study is required to support each of the proposal trafficking systems.

4.2 Proposed Model

By analysing the results obtained here and in the literature, we can propose a model for the intracellular transport of HIV-1. hnRNP A2 may bind the A2RE-containing transcripts in the nucleus and then, due to the high concentration of hnRNP A2 in the nucleus, it will dimerize as suggested in the hnRNP A2 trafficking system. This event, with the cooperation of other cellular proteins and Rev [172], will allow the transport of the trancripts out of the nucleus. Once in the cytoplasm, the dimerized hnRNP A2 molecules will be disassociated, due to the low hnRNP concentration in the cytoplasm compare with the nucleus. However, formation of RNP complex will allow several molecules of hnRNP A2 protein to bind to the A2RE element to aggregate together. Different cellular proteins, such as TOG2 and kinesin, allow transport and propel the complex along the microtubules, in an anterograde route, toward the plus-end. Once at the proximity of the cell membrane, the RNP complex may interact with the endocytic recycling compartment to favour the transfer of the RNA from the RNP to the Rab recycling vesicles and ensure the presence of the viral RNA at the plasma membrane, since the vesicle are directed to it. Those vesicles would then transport the RNA to the cell plasma membrane. In parallel, Gag may also interact with the genomic RNA, at the centrosome, and participate in the transport of RNA to the cell surface, in a microtubule dependent pathway.



4.1 Proposed Model for Intracellular Transport of Genomic RNA Mediated by hnRNP A2

hnRNP A2 may bind the A2RE-containing transcripts in the nucleus and then, it will dimerize. In cooperation with other cellular proteins and Rev, hnRNP A2 may help the transport of the transcripts out of the nucleus. In the cytoplasm, the dimerized hnRNP A2 molecules will be disassociated. However, formation of RNP complex will allow several molecules of hnRNP A2 protein to bind to the A2RE element to aggregate together. Different cellular proteins, such as TOG2 and kinesin, allow transport and propel the complex along the microtubules, in an anterograde route, toward the plus-end. At the proximity of the cell membrane, the RNP complex may interact with the endocytic recycling compartment to the transfer of the RNA from the RNP to the Rab vesicles. Those vesicles would transport the RNA to the cell plasma membrane. Gag may also interact with the genomic RNA, through the centrosome, and participate in the transport of RNA to the cell surface, in a microtubule independent pathway.

4.3 Future direction and conclusion

In order to fully understand, the role of hnRNP A2 in the HIV-1 RNA trafficking, many other experiments would have to be done. First, we would need to examine the different phenotype observed previously, where the genomic RNA was confined to the nucleus. The difference may reside in the system used. In fact, earlier data indicates that expression of the HxBru A2RE 2-1 results in a nuclear accumulation, however, in our system under hnRNP A2 knockdown, a perinuclear accumulation of the genomic RNA is observed. Presence of a mutation in the HIV-1 genome may then abolish other potential interactions, with other proteins that may play an important role in the trafficking.

One important aspect to explore is the potential effect of the increase presence of the viral RNA in the viral particles. Since it was already demonstrated that viral content is important in viral infectivity and that an increase of the viral contain impairs the viral viability, an experiment measuring the infectivity should be accomplished. Futhermore, it would be interesting to study the effect of the RNA increase on the different viral replication steps (such as the decapsidation, reverse transcription, and nuclear importation), since this may have an impact on viral infectivity. Also, it would be interesting to look if the PIC trafficking to the nucleus, as well as the nuclear import step, is affected. This could be accomplished through infection of cells by wild-type virus or by virus obtained from HxBru+siRNA hnRNP A2 treated cells. This may then lead to a potential role of hnRNP A2 in the early event of the replication cycle. Development of a continuously-expressing -hnRNP A2 lymphocytes T cell may be useful since such it would help in confirming the data and would be a pathophysiologicaly relevant system.

In conclusion, the data presented here demonstrated the important role of a cellular protein in HIV-1 RNA localisation. Since it may interacts will critical motor and regulator proteins involved in the transport in general, the data observed here revealed a role of this protein in the cytosolic trafficking pathway of HIV-1 genomic RNA localisation. Moreover, the effect of a depletion of hnRNP A2 indicates that hnRNP A2 is involved in RNA encapsidation. This may primo-impact on viral infection and

pathogenesis. We propose that here hnRNP A2 may possibly act as a bridge between the RNP complex and the microtubule network. Finally, virus-host interaction are critical to viral pathogenesis. Actually, attempts are being made to elaborate on new targets that are based on these events and, finally, hnRNP A2 function in RNA trafficking may be part of the next generation of drug treatments.

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CONTEX ENVIRONNEMENT

certifie que ***Kathy Lévesque***

a réussi avec succès une session de formation
d'une durée de 6:00 heures

PRINCIPES DE RADIOPROTECTION

Lady Davis Institute - Principes de radioprotection
Cours du 03-06-23

1. Introduction aux rayonnements et structure de la matière
2. Unités de rayonnement.....
3. Rayonnement et radioactivité.....
4. Détection et mesure du rayonnement.....
5. Effets biologiques du rayonnement.....
6. Effets des rayonnements sur le fœtus.....
7. Exigences réglementaires et limites d'exposition.....
8. Contrôle de la radioprotection.....
9. Procédures d'exploitation et d'urgence.....
10. Exigences en matière de transport.....

Certificat émis le 26 juin 2003